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(54) Title: PURIFIED MULTIMERASE

(57) Abstract

There is disclosed a purified multimerase having an indirect and a direct proteolytic activity, which converts vWF having a singlet structure to vWF having a satellite structure and is active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, as well as a method of preparing the multimerase according to the invention.

PURIFIED MULTIMERASE

The invention relates to a preparation comprising von
Willebrand factor protease, a method of preparing the same as
well as a method of preparing von Willebrand factor with
satellite structure.

Von Willebrand factor (vWF) is a glycoprotein circulating in
plasma as a series of multimers ranging in size from about 500
to 20,000 kD. Multimeric forms of vWF are composed of 250 kD
polypeptide subunits linked together by disulfide bonds. vWF
mediates the initial platelet adhesion to the subendothelium of
the damaged vessel wall, only the larges multimers also
exhibiting a haemostatic activity. It is assumed that
endothelial cells secrete large polymeric forms of vWF and that
those forms of vWF which have a low molecular weight (low
molecular weight vWF, LMW) have arisen from proteolytic
cleavage.

The multimers having large molecular masses are stored in
the Weibel Palade bodies of the endothelial cells and are
liberated upon stimulation.

vWF can bind blood coagulation factor VIII, thus forming the
factor VIII complex or factor VIII:C/vWF complex which contains
factor VIII:C as a stabilized protein. A vWF deficiency
necessarily also will lead to a reduction in the factor VIII:C
concentration in blood, since the stabilizing effect of vWF is
missing.

The proteolytic degradation of vWF is a physiological
process in healthy individuals, yet in patients suffering from
von Willebrand disease (vWD) type 2A it may be accelerated, and
as a consequence these patients lack the vWF multimers with the
largest molecular masses.

A small portion of the vWF present in normal plasma
circulates as 189, 176 and 140 kD fragments resulting from
proteolytic degradation of vWF in vivo, the 140 kD fragment
being derived from the N-terminal region, and the 176 kD
fragment from the C-terminal region of the subunit. LMW forms of
vWF were isolated from normal human plasma and subjected to SDS-
PAGE after disulfide reduction. The unusually high portion of
vWF fragments is compatible with the view that the LMW forms of

vWF have been partially or predominantly derived from large
multimers by proteolytic degradation.

The infusion of 1-desamino-8-D-arginine vasopressin in
normal subjects was followed by the appearance in plasma of
multimers of large molecular masses that were subsequently
rapidly cleaved through proteolytic degradation. A lack of large
vWF multimers and an increased level of proteolytic fragments
were also observed in acquired von Willebrand disease (vWD)
associated with myeloproliferative syndrome, indicating
increased in vivo proteolysis in this condition as well.
Unusually large molecular forms of vWF were found in patients
with thrombotic thrombocytopenic purpura (TTP); these large
multimers disappeared after transfusion with normal fresh frozen
plasma. It is evident that proteolytic enzyme(s) are involved
in the physiological regulation of the polymeric size of vWF in
the circulating blood and that they also play an important role
in the pathogenesis of vWF abnormalities in some patients with
congenital or acquired disorders of hemostasis; however, the
proteolytic enzyme responsible for vWF proteolysis in normal
human plasma has not yet been identified.

Several proteases have been shown to be able to cleave vWF,
thereby impairing its binding affinity for platelets; however,
treatment of vWF with these proteases in each case resulted in
cleavage products different from the fragments derived from in
vivo cleavage.

Thus, e.g., plasmin is capable of cleaving several peptide
bonds in vWF, yet there remains a high molecular weight core
region retaining about 70% of the platelet agglutinating
activity (determined as ristocetin cofactor). A 34 kD peptide
was split from the N-termini of individual vWF subunits in the
early stage of the treatment with plasmin. Epitope mapping of
plasmin-induced fragments clearly showed that these fragments
originated from regions of the vWF subunit that were different
from the fragments present in circulating plasma.

Porcine pancreatic elastase and various serine proteases
released from human leukocytes were shown to degrade vWF
proteolytically with a resultant loss of large multimers.
Epitope mapping of the degradation products again indicated that
these fragments also differed from those present in normal

plasma and in vWD type 2A. Moreover, vWF multimeric patterns in plasma samples from patients with extremely high or low neutrophil counts were not significantly different from those in normal human plasma.

In addition to the above serine proteases, a calpain-like protease released from human platelets was also shown to degrade large vWF multimers.

Furthermore, analysis of the circulating vWF fragments indicated that the peptide bond between amino acid residues 842Tyr and 843Met is cleaved in the vWF subunit of patients with vWD of type 2A; a cleavage site indicating a calpain-like specificity. However, it could also be shown that calpains from porcine erythrocytes and porcine kidney in vivo failed to generate the vWF fragments produced.

Recombinant vWF (r-vWF) can be produced in CHO cells, e.g. according to FEBS Letter 375, 259-262 (1995). The r-vWF recovered in this manner is available as a mature vWF and has a singlet structure, i.e. it differs from plasmatic vWF, which always has a characteristic satellite structure when examined on 2% SDS agarose gels.

In WO 96/10584 it is described that the r-vWF is comprised of multimers with high structural integrity which is retained even after purification and treatment for the inactivation of viruses. The intact structure of the r-vWF is defined by a result of electrophoretic analysis consisting of multimer bands with an absence of satellite bands.

To prepare an r-vWF preparation having a structure corresponding to that of plasmatic vWF from the r-vWF with singlet structur, a treatment with a physiological vWF protease activity is thus necessary.

Thus it is the object of the present invention to provide an enzyme activity which is capable of proteolytically processing vWF in a physiologic manner and optionally to degrade the same, and, respectively, to provide a preparation which comprises such an enzyme activity.

Since the pathologic symptoms due to abnormal vWF degrading activities or due to an increased vWF level may often cause thromboses or thromboembolic disorders, it is a further object of the present invention to re-adjust the normal levels by a

pharmaceutical preparation comprising a physiological vWF-
cleaving enzyme activity.

According to the invention, these objects are achieved by a
protein comprising an enzyme activity purified according to the
present specification, which is named "multimerase" because of
its proteolytic activity relative to von Willebrand factor
multimers and has the following properties:

a) it has an indirect or a direct proteolytic activity and
converts vWF having a singlet structure to vWF having a
satellite structure,

b) it is active in the presence of the serine protease
inhibitor diisopropyl fluorophosphate (DFP) or of the calpain
protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$.

Property a) can be determined by a simple test in which a
recombinant vWF having singlet structure is treated with the
multimerase, which test is explained in more detail in the
Examples, and this property is a completely new property of
proteases and so far is unique to the multimerase according to
the invention. The satellite structure, e.g. triplet structure,
corresponds to a multimer pattern after electrophoretic analysis
of plasmatic vWF which is characterized by central bands and
several satellite bands (cf. Figs. 12 and 13).

The terms "direct" and "indirect" indicate that the
multimerase according to the invention has direct proteolytic
activity mediated without an activator, or that it contributes
to enhanced proteolytic activity via an appropriate activating
effect or a mediator.

Preferably, the multimerase has an optimum proteolytic
activity at a pH ranging from 7 to 10, preferably at a pH of
from 7.5 to 8.5. However, this optimum pH range is largely
dependent on the (ionic) environment in which the reaction is
carried out.

Preferably, the multimerase is provided in a fraction
obtained upon gel filtration of a plasma or serum fraction
corresponding to the molecular weight of more than 200 kD,
preferably around 300 kD.

Preferably, after a purification according to the invention,
the multimerase is provided in at least 1,000-fold, preferably
at least 10,000-fold enriched form as compared to plasma.

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Preferably, the purified multimerase has a specific activity
of at least 10 U/mg protein in the presence of a serine protease
inhibitor. One unit is defined as that enzyme activity which is
contained in one milliliter of human normal plasma or can be
generated therefrom, respectively.

It has been shown that the multimerase is particularly
active in the presence of or after incubation with,
respectively, bivalent metal ions, such as alkaline earth ions,
in particular Ba^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+}, and that its activity
can be further increased by shear stress, reduced ionic strength
or chaotropic agents.

The activity of the multimerase is especially generated if
the ionic strength of the environment is lowered to below the
physiologic ionic strength, in particular corresponding to a
concentration of less than 15 mM Tris. The shearing forces which
may act when flowing through a capillary, when stirring in
containers or when passing through a nozzle or generally on
account of a mechanical action on the multimerase or its
substrate, respectively, substantially contribute to the
increased activity of the multimerase. It is presumed that the
effect of chaotropic substances is due to the partial or
complete change of the tertiary structure of a protein. These
substances include salts, such as ammonium sulphate, calcium
chloride, thiocyanates, but also urea and guanidinium chloride.

Preferably, the multimerase preparation according to the
invention has an increased activity relative to modified vWF,
such as partially denatured vWF or vWF having a conformation
change.

The multimerase according to the invention can be inhibited
by not effecting or stopping activation. Thus, chelating agents,
such as EDTA, EGTA and citrate, which have an inhibiting effect
on the metalloenzyme, can be used as inhibitors. The activity of
the multimerase may, however, also be inhibited by vWF peptides
corresponding to the Tyr842-Met843 peptide sequence which can
block the binding site of the multimerase. Lowering of the
temperature, e.g. by freezing the multimerase-containing
fraction, also results in a reduction of the multimerase
activity.

In a further aspect, the present invention relates to a

method of purifying the multimerase according to the invention
from plasma, serum, or from a fraction of plasma or serum, by a
chromatographic procedure, the purified multimerase being
recovered from those fractions in which the proteolytic activity
is found for the inactivation of vWF in the presence of a serine
protease inhibitor, preferably aprotinin.

The invention also relates to a preparation comprising a
1,000-fold, preferably 10,000-fold enriched multimerase as
compared to plasma.

The present invention also relates to a method of producing
a vWF having a satellite structure, by incubating a vWF having a
singlet structure with a multimerase according to the invention,
whereby a vWF having a satellite structure is obtained and the
vWF is recovered.

The method is preferably carried out under nearly
physiological conditions. For instance, the conversion of the
vWF is effected at a pH ranging from 7 to 10, preferably 7.5 to
8.5, and at a temperature of from 20 to 40°C, preferably
approximately room temperature, for a period of time which
suffices for the formation of satellite bands without
substantially affecting the activity of the vWF.

For this preparation of the vWF having satellite structure,
preferably a multimerase is used which, as described before,
develops an optimum activity under certain conditions, e.g. by
pre-incubation with metal ions. The duration of the reaction
also depends on the enzyme/substrate ratio, which is selected in
a range of from 0.01:1 to 100:1, preferably within a range of
from 0.1:1 to 10:1, most preferred is the physiological ratio of
approximately 1:1 (unit per unit). The reaction is either
stopped by the addition of a suitable inhibitor, in particular
of a chelating agent, or by lowering the temperature, e.g. by
freezing the reaction solution, or by stopping the reaction by
not generating the activity. The course of the reaction can be
monitored by electrophoretic analysis, and a suitable end can be
determined.

This preparation of the vWF having satellite structure can
be effected both in vitro in a suitable reaction environment,
and in vivo or ex vivo, e.g. by the expression of r-vWF in a
cell culture or extracorporeally.

Incubation of the multimerase with the biologically active vWF is preferably carried out in the presence of a serine protease inhibitor, since by the incubation in the presence of a serine protease inhibitor, unspecific proteolytical processes are avoided which are not directly related to the multimerase and which are caused by serine proteases present in blood or in plasma. The proteolytic activity of a possibly contaminating serine protease can be avoided under the protection of such an inhibitor.

Preferably, the multimerase according to the invention is thus obtained in a fraction which is substantially free from a serine protease activity, corresponding to a plasmin activity of less than the detection limit of a test by using a specific chromogenic substrate, e.g. S2251 according to EP-0 353 218.

In another aspect, the present invention relates to a pharmaceutical composition comprising a purified "multimerase" activity according to the present invention which has the following properties:

a) it has an indirect or a direct proteolytic activity and converts vWF having a singlet structure to vWF having a satellite structure,

b) it is active in the presence of the serine protease inhibitor diisopropyl fluorophosphate (DFP) or of the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, and

c) it is treated for inactivation or removal of viruses. For properties a) and b): see above.

The virus inactivation or removal treatment may be performed by any treatment accepted as being efficient. According to preferred embodiments of the present invention, the pharmaceutical composition comprising the multimerase activity is treated with tensides and/or heat, e.g. by a heat treatment in the solid state, especially a steam treatment according to EP-0 159 311, or EP-0 519 901, or EP-0 674 531.

Further treatments for inactivation of viruses also comprise treatment by chemical or chemical/physical methods, e.g. with chaotropic agents according to WO94/13329, DE 44 34 538 or EP-0 131 740 (solvents), or photoinactivation.

Nanofiltration also represents a preferred method of

depleting viruses within the scope of the present invention.

According to a further aspect, the present invention relates to the multimerase according to the invention as a drug, in particular the use of the multimerase according to the invention for the prevention and therapy of thromboses or thromboembolic disorders, preferably for the prevention and therapy of a supranormal vWF level or of an increased level of high-molecular vWF by administering to a patient an effective dose of a multimerase-containing pharmaceutical preparation.

This supranormal vWF level may be caused by a supranormal vWF antigen concentration or a supranormal vWF activity, activity in particular referring to the primary hemostatic activity, but also to the binding activity to the subendothelium, to the thrombocytes, to the thrombocyte-adhesion proteins, such as GPIb and GPIIb/IIIa complex, to collagen, to factor VIII and to heparin.

Preferably, the present invention relates to the prevention and therapy of thrombotic thrombocytopenic purpura, Henoch-Schönlein purpura, hemolytic-uremic syndrome and preeclampsia or neonatal thrombocytopenia.

Thrombotic thrombocytopenic purpura (TTP) was first described by Moschcowitz, 1924, and is characterized by thrombocytopenia with normal megakaryocyte number in bone marrow, microvascular thromboses, hyaline thrombi, restricted function of the kidneys and endothelial cell proliferation. The pathological and clinical results in patients suffering from TTP suggest a direct thrombocyte-aggregating stimulus in the microcirculation.

In addition to thrombocytopenia, the clinical picture includes intravascular hemolysis with fragmented erythrocytes and neurological symptoms. Incidence in the normal population is estimated at 0.1/100,000/year. In HIV infected individuals, it is approximately 4/100,000. Different types of TTP seem to exist. Beside a primary TTP, also so-called secondary TTPs are found in connection with pregnancy, chemotherapy, bone marrow transplantation and autoimmune diseases. With chronic relapsing TTP (CRTTP), there are frequent episodes at regular intervals. Unusually high multimeric forms of vWF which are liberated by endothelial cells are found in plasma from patients afflicted

with TTP. Under "shear stress", these unusually large vW
multimers bind to glycoprotein Ib and glycoprotein IIb/IIa much
more strongly than normal vWF and cause the intravascular
thrombocyte aggregation.

Attacks of TTP are difficult to treat. In most instances,
the aim is to remove the high-molecular vWF multimers by plasma
exchange.

Henoch-Schönlein purpura (HSP) is a clinical syndrome
characterized by non-thrombocytopenic purpura, arthralgias and
glomerulonephritis. Patients afflicted with HSP have an abnormal
vWF multimer picture with supranormal high molecular weight vW
multimers. Detection of these supranormal vW multimers in HSP
suggests an impaired endothelial cell function. Multimers of low
molecular weight are delivered by the endothelial cells in a so-
called constitutive mechanism, while the high molecular weight
multimers are liberated from the "Weibel/Palade bodies" of the
endothelial cells upon stimulation.

The pharmaceutical preparation according to the invention is
produced by purifying a multimerase-containing starting
material, preferably by chromatographic methods, such as ion
exchange chromatrography, hydrophobic chromatography or affinity
chromatography, and subsequently finishing it by known methods,
optionally by combining it with suitable buffer, auxiliary,
preserving and/or stabilizing substances or protease inhibitors,
respectively, and filling it into containers into a form
suitable for administration, and preferably packing it so as to
be storage-stable, optionally in the lyophilized or frozen
state.

The effective dosage of the preparation when applied will
vary, depending on the respective syndrome, and preferably
should be chosen after determination of the endogenous vWF
protease activity in the patient [e.g. by means of the
determination method according to the invention (infra)]. The
dosage also depends on whether or not the parenteral, preferably
intravenous, subcutaneous or intramuscular administration is to
be effected in bolus form, and whether it is to be effected
systemically and/or locally at the site of thrombosis.

When administering the preparation according to the
invention, the vWF plasma concentration as well as the structure

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of the vWF and the vWF protease activity in the patient should
be monitored, and dosage should be optimized on the basis of
these data.

   The preparation according to the invention may be produced
both by purification from blood, serum or plasma, and by a
respective expression system.

   A preparation according to the invention may also be
provided by expression of the multimerase in vivo or ex vivo.
Suitable for this are above all cells derived from mammals, in
particular human cells, which can be cultured and optionally
introduced into the patient. Within the scope of a gene therapy,
it is possible to insert the nucleic acid encoding the
multimerase into the cells, in particular into arterial
endothelial cells, which are capable of expressing the
multimerase in vessels or in vessel prostheses, respectively.

   For this technology, a nucleic acid encoding the multimerase
can be provided in a known manner. A pre-requisite is a purified
multimerase which can be used as a template for the nucleic
acid. This nucleic acid may be inserted either via a vector or
directly into the host cell, where expression of the multimerase
is effected in a known manner.

   Preferred chromatographic purification methods are ion
exchange chromatography, affinity chromatography or gel
filtration, combinations of these methods and multiple
chromatographies being considered particularly preferred.

   Yet another aspect of the present invention relates to a
method of assaying the proteolytic activity of the multimerase
according to the present invention. To detect the multimerase
activity, a method is used which comprises the following steps:
incubating a fraction containing the multimerase with a vWF, and
determining the reaction rate of vWF. The fraction is, for
instance, a plasma sample or a plasma fraction or a fraction
with the enriched, possibly purified, multimerase. The latter is
contacted with a vWF which may be a native human vWF, preferably
having a singlet structure, or with a corresponding vWF fragment
containing the Tyr842-Met843 peptide sequence.

   Incubation is preferably effected under conditions which
ensure an optimum activity of the multimerase, as described
before. During the incubation which is preferably carried out

under nearly physiologic conditions, at pH 7 to 10, preferably
pH 7.5 to 8.5, at 20 to 40°C, preferably approximately room
temperature, vWF is reacted, degraded or inactivated,
respectively, and the result of the reaction can be determined.
What is detected are, e.g., vWF degradation products and
fragments, such as multimers of low molecular mass or satellite
bands after electrophoretic analysis, or the change of the
activity of vWF.

In the method according to the invention, incubation of the
multimerase with the biologically active vWF is preferably
carried out in the presence of a serine protease inhibitor, and
the reaction rate preferably is determined by the extent of
inactivation or by the formation of certain fragments or
satellite bands of the vWF.

By incubation in the presence of a serine protease
inhibitor, such as aprotinin, unspecific proteolytic processes
which cannot be directly traced back to the multimerase and
which are caused by serine proteases present in blood or plasma
can be avoided.

Determination of the proteolytic acitivity of the multimer
according to the invention may, however, not only be carried out
by direct conversion of the vWF, but also by cleavage of a
chromogenic substrate comprising a peptide sequence homologous
with the vWF peptide binding sequence Tyr842-Met843 as well as a
chromogenic group, by cleaving the substrate under the action of
the multimerase and forming a chromophore, whereupon colour
development is determined which can be viewed as directly
proportional to the proteolytic activity of the multimerase.

Furthermore, the invention also relates to vWF preparations
that have been produced free from multimerase activity and in
particular in the absence of a multimerase activity. These vWF
preparations preferably contain the native vWF which contains
the entire spectrum of the multimers and which is characterized
by a low degree of cleavage of the Tyr842-Met843 peptide bond.
Alternatively, however, a vWF fraction, vWF, a vWF derivative or
a vWF fragment or a vWF mutant can be contained, with the
proviso that it contain the Tyr842-Met843 peptide sequence. This
preparation above all is suitable for the production of
pharmaceutical preparations, since, due to the lack of

multimerase activity, they remain unchanged even after prolonged
storage, e.g. in the liquid state.

The vWF preparations according to the invention can be
provided by specific inhibition of the multimerase or by
separation or depletion of the multimerase by means of the
afore-mentioned chromatographic purification methods, in
particular by immunoaffinity chromatography by using antibodies
directed against the multimerase. Inhibition can be obtained by
a content of a chelating agent in the pharmaceutical preparation
or by specific inhibitors which bind like a "substrate mimic" to
the active center of the multimerase, thus inhibiting the
activity in competitive manner.

The pharmaceutical vWF preparation containing a reduced
multimerase activity, in particular of less than the detection
limit, preferably further comprises the blood coagulation factor
VIII, a factor VIII derivative or a factor VIII mutant,
stabilized by complex formation to the vWF. Since the multimer
pattern in the vWF preparation according to the invention
surprisingly remains unchanged even over an extended period of
time, i.e. is permanent, also the stabilizing effect for factor
VIII in the pharmaceutical preparaction according to the
invention remains unchanged and permanent even after an extended
storage in the liquid state.

The pharmaceutical preparation according to the invention
thus is storage-stable not only in the lyophilized or liquid-
deep-frozen states, but also as a liquid preparation.

By aid of the multimerase according to the invention, it is
furthermore possible to define resistent forms of the vWF.
Certain vWF fractions, derivatives or mutants are accordingly
incubated with a fraction containing a defined multimerase-
activity, and the extent of proteolytic degradation is
determined. A resistent form of vWF is present if the
multimerase activity does not substantially influence the vWF
activity (e.g. by altering the Tyr842-Met843 peptide sequence).
This resistent form of vWF can be utilized to stabilize FVIII:C
in vitro and in vivo. Thereby not only the half-life of the vWF
form in vivo is extended, but also the residence time of FVIII:C
in plasma.

Finally, the present invention also relates to a method of

producing antibodies against the multimerase according to the
invention, which is carried out in that a preparation containing
the enriched, optionally purified multimerase according to the
invention is used as an immunogen, and respective polyclonal or
monoclonal antibodies against the multimerase are produced in a
manner known per se.

Furthermore, the present invention also relates to an
antibody preparation containing antibodies against the
multimerase, wherein the antibody preparation may be monoclonal
or polyclonal.

Preferably, the antibodies according to the invention are
immobilized on a solid phase and may thus be used for
immunological detection or for the purification of the
multimerase.

The present invention will be explained in more detail by
way of the following examples and drawing figures, to which,
however, it shall not be restricted.

Fig. 1 shows the purification of vWF;

Fig. 2 shows the degradation of vWF by contaminating
proteases;

Fig. 3 shows the effect of salt and urea on the cleavage of
vWF;

Fig. 4 shows the degradation of vWF by various blood
components;

Fig. 5 shows the purification of the proteins from plasma;

Fig. 6 shows the chromatography of the protease on Sephacryl
S-300 HR;

Fig. 7 shows an SDS-PAGE of the fractions obtained after
Sephacryl S-300 HR chromatography;

Fig. 8 shows the influence of metal ions and pH on the
activity of the vWF-cleaving protease;

Fig. 9 shows the effect of protease inhibitors on the vWF
degradation;

Fig. 10 shows an SDS-PAGE of the vWF degradation products;

Fig. 11 shows the effect of vWF protease on fibrinogen, BSA,
collagen from calf skin and diluted normal plasma;

Fig. 12 shows the degradation of vWF by the multimerase
according to the invention;

Fig. 13 shows the degradation of vWF from cryoprecipitate

with the multimerase according to the invention;

Fig. 14 shows the degradation of vWF high multimers and r-vWF by means of the multimerase according to the invention;

Fig. 15 shows the degradation of r-vWF with plasmin;

Fig. 16 shows the degradation of r-vWF with plasmin with and without inhibitors;

Fig. 17 shows the degradation of vWF from cryoprecipitate with plasmin; and

Fig. 18 shows the incubation of r-vWF in vWF-deficient human plasma.

E x a m p l e s :

Purification of vWF

vWF was purified by gel filtration of human cryoprecipitate, obtained from 1 l citrated plasma, on a 2.6 x 35-cm column of Sepharose CL-2B (Pharmacia, Uppsala, Sweden). Elution was performed with 0.13 M sodium chloride, 0.01 M citrate, 0.01 M Tris-HCl, pH 7.4. Fractions of 6 ml were collected at a flow rate of 24 ml/h. vWF antigen was measured by ELISA by using polyclonal rabbit antiserum against human vWF (RAHu/FVIII, from Nordic (Tillburg, The Netherlands), peroxidase-labeled rabbit antihuman-vWF, mouse monoclonal antibody against rabbit IgG (M737), and the kit for immunostaining (K670) (all from Dako (Glostrup, Denmark)), according to the instructions included with the kit.

The results of vWF-purification are illustrated in Fig. 1: Fractions 12 to 20 contained vWF substantially free from contaminating proteins. These fractions were used as the substrate in determining the vWF cleavage activity (infra).

The vWF contained in these fractions proved to be very stable; even after a 24 h dialysis incubation at 37°C (in the absence of inhibitors), a multimer pattern identical to that of the untreated fractions was obtained, both, with the 24 h dialysis against 0.13 M sodium chloride, 0.01 M citrate, 0.01 M Tris-HCl, pH 7.4, and with the 24 h dialysis against 1 M urea, 5 mM Tris-HCl, pH 7.4. SDS agarose gels of these assays are illustrated in Fig. 2.

The subsequent fractions, however, showed a pronounced vWF degradation at low salt concentrations in the presence of urea.

Effect of the salt and urea concentrations on the cleavage of vWF:

Aliquots of the fraction No. 29 from the sepharose CL-2B column were dialyzed against a 5 mM Tris-HCl (pH 7.4) solution of varying salt concentrations, in the presence or absence of urea, no calcium ions being added. Parallel assays were performed with further aliquots of fraction 29 which had been subjected to a 5 minute incubation with 1 mM DFP prior to dialysis. From Fig. 3 it is apparent that the large vWF multimers disappeared after dialysis, if no sodium chloride was present. Moreover, the vWF degradation could be increased markedly by 1 M urea even at physiological salt concentrations. The combination of a low salt concentration with 1 M urea led to the complete degradation of vWF. A preincubation with 1 mM DFP, a strong serine protease inhibitor, yielded identical results. Thus, dialysis at 37°C against 1 M urea, 5 mM Tris-HCl was chosen as an optimum condition for a sensitive assay for determining the protease activity.

Assay of the vWF cleaving activity

Because, as has been mentioned, the degradation of vWF was found to be strongly dependent on the buffer conditions used, the enzyme and the substrate were incubated on a circulary dialysis membrane (Millipore VSWP; diameter, 25 mm; from Millipore (Bedford, USA)), the surface being incubated with 50 ml of dialysis buffer. The buffer system which had been sufficiently optimized for the instant assaying system thus comprised a dialysis solution with 1 M urea in 5 mM Tris-HCl at pH 8. Full activation of the protease was achieved by preincubation for 5 minutes at 37°C with 10 mM barium chloride. In a typical experiment, 50 µl activated protease solution and 100 µl substrate solution were carefully placed on a floating membrane, and incubated in a closed tube for 24 h in a dry oven at 37°C.

Protease-free fractions of vWF from the Sepharose CL-2B column were used as substrate solution, the vWF concentration in the incubation mixture was about 30 µg/ml. The reaction mixtures were then removed from the surface of the membrane filters and subjected to SDS-agarose gel electrophoresis to examine the multimeric pattern of vWF.

SDS-agarose gel electrophoresis and immunoblotting of unreduced vWF:

Thin-layer agarose electrophoresis was performed using the discontinuous buffer system as described by Ruggeri and Zimmerman (Blood 57 (1981), 1140). Before electrophoresis, each sample was incubated with an equal volume of the SDS-containing sample buffer for 15 minutes at 60°C. The horizontal electrophoresis was performed in 1% HGT(P) agarose (2 mm thick, 20 cm wide, and 8.5 cm long) for 17 h at 16°C in an LKB multiphor apparatus (Pharmacia-LKB) at 80V and 10 mA. The proteins were electrotransferred to a nitrocellulose membrane (from Schleicher & Schuell (Dassel, Germany) for 3 hours at 26 V and 1.4 A using a Trans-Blot cell from Bio-Rad with a buffer containing 0.04% SDS and 0.05 M phosphate, pH 7.4. vWF multimers were identified with peroxidase-conjugated antibodies against human vWF (with peroxidase-labelled rabbit-antihuman-vWF antibody P226 from Dako (Glostrup, Denmark)).

SDS-PAGE:

SDS-5% polyacrylamide gels were prepared according to Laemmli (Nature 227 (1970), 680). The proteins were reduced with 65 mmol DTT for 15 minutes at 60°C. Unreduced and reduced chromtatographic fractions from the Sephacryl S-300 HR column were electrophoretically separated in 3-mm-thick gels for 18 hours at 60 V and were stained with Coomassie Blue.

Immunoblotting of reduced vWF:

Reduced samples of undegraded and proteolytically cleaved vWF were subjected to SDS-PAGE and were electrotransferred to nitrocellulose as described above. For detection of vWF fragments, the nitrocellulose was incubated with a rabbit antiserum against human vWF (RAHu/FVIII), followed by incubation with mouse antibodies against rabbit IgG (M737; from Dako (Glostrup, Denmark)) and the APAAP (alkaline phosphatase anti-alkaline phosphatase) reagent (K670 from Dako (Glostrup, Denmark)) according to Furlan et al. (PNAS 90 (1993), 7503).

Preparation of blood components to be tested for the presence of the protease:

Whole blood was drawn from normal individuals into a glass tube without the addition of anticoagulants. After 15 minutes at room temperature, the clotted blood sample was centrifuged twice

for 15 minutes at 2,500g. Subsequently, 10 μl of 10 mM PPACK
(Diphenylalanyl- prolyl arginine-chloromethylketone from Bachem,
Bubendorf, (Switzerland)) was added to 10 ml serum, and the
sample was incubated for 10 minutes at room temperature and
frozen at -20°C. From the same subject, 90 ml of blood was drawn
into 10 ml of 0.13 M sodium citrate in a plastic bottle. After
centrifugation for 15 minutes at 300g, about 50 ml platelet-rich
plasma (PRP) was recovered. One aliquot of PRP was centrifuged
for 15 minutes at 2,500g. The resulting platelet-poor plasma
(PPP) was recentrifuged for 15 minutes at 3,000g and frozen at -
20°C. The platelet sediment was resuspended in 0.9% sodium
chloride in 1/15 of the original volume and subjected to 3
cycles of freezing and thawing. The lysed platelets were
centrifuged for 15 min at 3,000g, and the resulting supernatant
and the sediment were frozen at -20°C. A frozen aliquot of the
PPP was slowly thawed to produce the cryoprecipitate. After
centrifugation for 15 minutes at -5°C and 3,000g, supernatant as
well as cryoprecipitate, taken up in 1/15 of the original volume
of 0.9% sodium chloride, were frozen at -20°C. Another aliquot
of PPP was transferred to a glass tube, mixed with 1/40 volume
of a 1 M calcium chloride solution and incubated for 15 minutes
at 37°C. After removal of the fibrin clot and 15-minute
centrifugation at 3,000g, PPACK was added to a final
concentration of 25 μM, and the defibrinated plasma was frozen
at -20°C. The frozen samples were incubated for 10 minutes each
at 37°C before the assay for determining the protease activity.

Assaying prepared blood components for protease activity:

10 μl of 10 mM PPACK and 10 μl of 0.55 M calcium chloride
was each admixed to 200 μl serum, PPP, cryoprecipitate-free PPP,
defibrinated PPP as well as 15-fold concentrated cryoprecipitate
and lysed platelets. After incubating for 10 minutes at 37°C, 10
μl aliquots were mixed with 40 μl vWF solution, dialyzed against
1 M urea, 5 mM Tris-HCl (pH 7.4) over night at 37°C, and the
proteolytic degradation of vWF detected by SDS agarose gel
electrophoresis.

It was found that protease activity was considerably higher
in PPP than in the supernatant or the sediment of lysed
platelets obtained from a 15-fold concentration of PRP (cf. Fig.
4). The protease activity was not affected by defibrination and

was partially recovered in the cryoprecipitate of PPP; the
cryoprecipitate corresponding to a 15-fold concentrated PPP
showed considerable activity, but the nonconcentrated
cryoprecipitate contained much less protease than did the
equivalent amount of PPP. There was no significant difference in
the protease activity found in citrated PPP and in serum
obtained from non-coagulated blood.

Purification of protease from plasma:

Fibrinogen was removed from plasma before chromatographic
procedures were performed, because these purification procedures
might induce activation of the coagulation cascade. Experiments
also indicated that the activity of the vWF-degrading protease
was not - or not substantially - affected by defibrination of
plasma or of the plasma fraction, DFP or PPACK.

Blood (450 ml) from healthy individuals was collected into
63 ml citrate/phosphate/dextrose/adenine (CPD-A$_1$) solutions on a
shaking balance. After 2 centrifugations for 15 minutes at 20°C
and 2,500g, 1 M calcium chloride was added to a final
concentration of 25 mM, and the recalcified PPP was stirred for
30 minutes at 37°C. After removal of the fibrin clot by
centrifugation, PPACK and DFP were added to the resulting serum
to final concentrations of 5 µM and 2 µM, respectively, and
incubated for 15 minutes at 37°C to inhibit the activated
clotting enzymes. Moreover, the serum was dialyzed against the
equilibrating buffer with 1 M sodium chloride and 0.05 M Tis-HCl
at pH 7.4. 50 ml aliquots were stored at -20°C until
purification.

The protease could first be purified from the defibrinated
normal plasma as starting material on Cu^{2+}-loaded chelating
Sepharose (1.6 x 22 cm; Pharmacia LKB) using step-wise elution
with equilibrating buffer containing increasing glycine
concentrations. Protease-containing fractions (cf. horizontal
line in Fig. 5A) were pooled (this pool containing 14.7% of the
original protein), dialyzed against 0.6 M $(NH_4)_2SO_4$/0.02 M Tris-
HCl, pH 7.4, and applied onto a column filled with butyl
sepharose (1.6 x 27 cm; Pharmacia LKB), most of the
contaminating proteins being removed by step-wise elution at a
lower $(NH_4)_2SO_4$ concentration. Proteolytically active fractions
(cf. transverse line in Fig. 5b) from two butyl sepharose

columns were pooled (this pool containing nearly all the
protease activity, yet only 0.75% of the original protein),
dialyzed against 1 mM EDTA, lyophilized, taken up in 5 ml
distilled water and applied on a Sephacryl S-300 HR column (2.6
x 90 cm; Pharmacia, LKB) that had been equilibrated with 0.15 M
NaCl, 0.01 M Tris-HCl, pH 7.4 (cf. Fig. 5C; the fractions below
the cross-section contained the entire protease activity, yet
only 0.08% of the original total protein).

To improve the resolution of gel filtration, a long column
was simulated by closing the first elution cycle, by
reintroducing the eluted proteins into the same column and
collecting them in a second cycle. Again, the active fractions
were pooled, dialyzed against 1 mM EDTA, lyophilized,
redissolved in 3 ml distilled water and submitted to a further
gel filtration on a Sephacryl S-300 HR column.

In this gel filtration, the first two cycles were closed and
the fractions were collected in the third cycle. In all these
depicted chromatographic procedures, fractions of 6 ml were
collected at a flow rate of 60 ml/h. Fractions containing the
protease were stored at -20°C.

The results are illustrated in Fig. 6 and show the elution
of the UV-absorving material (Fig. 6A) and of the protease
activity (Fig. 6B). The peak of the protease activity (fractions
9 to 17) contained merely 0.009% of the original serum protein.
Thus, a purification factor of approximately 10,000 was reached.
Nevertheless, the protease preparation obtained still contained
substantial amounts of contaminating proteins (cf. Fig. 7). The
peak of the protease activity (fractions 11 to 15) occurred
together with a protein band in the unreduced gel with a
molecular weight of approximately 300 kD, which appeared with
several proteins with a molecular weight ranging between 130 and
450 kD (main bands with M_r of 450, 200, 180 and 130 kD).

Amino acid composition and sequence analysis:

For analysis of the amino acid composition and of amino acid
sequence, the unreduced peak fraction of the protease and the
reduced vWF fragments were dialyzed against 1 mM EDTA, were
lyophilized, and were redissolved in 1/50 of the original volume
before SDS-PAGE. The HMW protein bands were electrotransferred
from the gel to a PVDF (polyvinyliden-difluoride) membrane

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(BioRad) for 6 hours at 26 V and 0.4 A, using 0.05% SDS, 10% methanol, 0.05 M boric acid, pH 9,0, as transfer buffer. After transfer, the membrane was stained with Coomassie-Blue in 40% methanol, destained in 40% methanol and 10% acetic acid, and air-dried. The excised bands were transferred to the blot cartridge of the Applied Biosystems Model 477A Sequenator, which was equipped with on-line high-performance liquid chromatography for analysis of phenylthiohydantoin derivatives. For analysis of amino acid compositions, the protein bands were hydrolyzed in the gas phase over 6N HCl for 22 hours at 110°C. The amino acids were extracted from the PVDF membrane with 70% 0.1 N HCl/30% methanol and were determined by high-performance liquid chromatography as phenylthiocarbamyl derivatives. The amino acid composition of the unreduced protein band coeluted from the Sephacryl S-300 HR column together with the protease activity is shown in Table 1.

Table 1

Amino Acid	Residues per 1,000 Residues
Asp + Asn	99.4
Thr	59.4
Ser	63.3
Glu + Gln	150.2
Pro	65.5
Gly	75.9
Ala	86.5
Val	70.7
Ile	32.9
Leu	101.7
Tyr	30.6
Phe	41.7
His	22.4
Lys	41.6
Arg	49.3

Influence of metal ions and pH on the activity of the vWF-cleaving protease:

Aliquots (95 µl) of the purified protease were preincubated for 15 minutes at 37°C with 5 µl of 0.2 M solutions of the following salts: $ZnCl_2$, $CuSO_4$, $Cd(CH_3COO)_2$, $CoSO_4$, $NiCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $SrCl_2$ and $BaCl_2$. Then, 50 µl of the purified vWF was added to each aliquot, and the incubation mixtures were transferred onto floating membrane filters. After incubation for 24 hours at 37°C against 1 M urea, 5 mM Tris-HCl, pH 7.4, the samples were removed from the filters and applied to SDS-agarose gel electrophoresis.

The protease showed no activation by Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+}. Only slight activation by Mg^{2+} was found; substantial activation could be obtained by Ca^{2+}, Sr^{2+}, and in particular by Ba^{2+} (cf. Fig. 8A).

Prolonged preincubation with calcium, strontium and barium was associated with a loss of protease activity even at physiological salt concentrations and in the absence of urea, probably because of autodigestion of the protease. In the absence of these ions the protease was quite stable in solution, and thus it was possible to purify the enzyme by chromatographic procedures taking place for several days at room temperature.

pH-optima of the protease after activation by calcium and barium were determined by preincubating the protease with 10 mM calcium chloride or with 10 mM barium chloride and incubating with vWF during the dialysis in urea Tris-HCl buffer, several buffers having a pH ranging between 6 and 11 being used. The multimer pattern of vWF in the dialyzed samples were analyzed with SDS agarose electrophoresis.

The pH optimum for vWF degradation by the calcium-activated protease was found at 9 to 10, whereas the highest activity of the barium-activated protease was at approximately pH 8 (cf. Fig. 8B).

The experiments for determining the vWF-degrading protease activity thus were always performed at pH 8 after an incubation with 10 mM barium chloride.

Effect of protease inhibitors on vWF degradation:

Purified protease was reactivated with 10 mM barium chloride for 5 minutes at 37°C and subsequently incubated for 15 minutes

at 37°C with the following protease inhbitors: EDTA (final
concentration 10 mM), EGTA (10 mM), Na$_3$-citrate (10 mM)
iodoacetamide (IAA, 10 mM), N-ethylmaleimide (NEM; 10 mM), DFP
(1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), N-α-p-tosyl-
L-lysine-chloromethylketone (TLCK; 1 mM), N-α-p-tosyl-L-
phenylalanine-chloromethylketone (TPCK; 1 mM), leupeptin (0.01
mM) and aprotinin (0.01 mM).

Furthermore, the following carbobenzyloxy-(Z)-peptidyl-
diazomethylketone inhibitors were tested (all at a final
concentration of 0.1 mM during preincubation with barium-
activating protease): Z-Leu-Leu-Tyr-CHN$_2$, Z-Val-Val-Tyr-CHN$_2$, Z-
Phe-Ala-CHN$_2$, Z-Phe(I)-Ala-CHN$_2$, Z-Tyr-Ala-CHN$_2$ and Z-Phe-Phe-
CHN$_2$.

After the preincubation, 100 μl aliquots of the enzyme
inhibitor mixtures were added to 50 μl of purified vWF solution,
and the mixtures were dialyzed against 1 M urea, 5 mM Tris-HCl,
at pH 7.4 for 24 hours at 37°C. The degradation of vWF was
assayed with SDS agarose electrophoresis and immunoblotting (cf.
Fig. 9A).
-tosyl-L-lysine-chloromethylketone (TLCK; 1 mM), N-α-p-tosyl-L-
phenylalanine-chloromethylketone (TPCK; 1 mM), leupeptin (0.01
mM) and aprotinin (0.01 mM).

Furthermore, the following carbobenzyloxy-(Z)-peptidyl-
diazomethylketone inhibitors were tested (all at a final
concentration of 0.1 mM during preincubation with barium-
activating protease): Z-Leu-Leu-Tyr-CHN$_2$, Z-Val-Val-Tyr-CHN$_2$, Z-
Phe-Ala-CHN$_2$, Z-Phe(I)-Ala-CHN$_2$, Z-Tyr-Ala-CHN$_2$ and Z-Phe-Phe-
CHN$_2$.

After the preincubation, 100 μl aliquots of the enzyme
inhibitor mixtures were added to 50 μl of purified vWF solution,
and the mixtures were dialyzed against 1 M urea, 5 mM Tris-HCl,
at pH 7.4 for 24 hours at 37°C. The degradation of vWF was
assayed with SDS agarose electrophoresis and immunoblotting (cf.
Fig. 9A).
-tosyl-L-phenylalanine-chloromethylketone (TPCK; 1 mM),
leupeptin (0.01 mM) and aprotinin (0.01 mM).

Furthermore, the following carbobenzyloxy-(Z)-peptidyl-
diazomethylketone inhibitors were tested (all at a final
concentration of 0.1 mM during preincubation with barium-

activating protease): Z-Leu-Leu-Tyr-CHN$_2$, Z-Val-Val-Tyr-CHN$_2$, Z-Phe-Ala-CHN$_2$, Z-Phe(I)-Ala-CHN$_2$, Z-Tyr-Ala-CHN$_2$ and Z-Phe-Phe-CHN$_2$.

After the preincubation, 100 µl aliquots of the enzyme inhibitor mixtures were added to 50 µl of purified vWF solution, and the mixtures were dialyzed against 1 M urea, 5 mM Tris-HCl, at pH 7.4 for 24 hours at 37°C. The degradation of vWF was assayed with SDS agarose electrophoresis and immunoblotting (cf. Fig. 9A).

The chelating agents EDTA and EGTA could inhibit the barium-preincubated protease completely, whereas with citrate only a partial inhibition could be attained. A 15-minute preincubation with sulfhydryl enzyme inhibitors IAA and NEM did not result in protease inhibition. There was also no inhibition of the protease according to the invention by serine protease inhibitors DFP, PMSF and aprotinin, or by serine/sulfhydryl protease inhibitors TLCK, TPCK and leupeptin.

In further experiments in which the same inhibitors were also incorporated into the dialysis solution, the same results were obtained, with one exception: with NEM a partial protease inhibition could be achieved.

Thus, it appears that the protease is very slowly inhibited by NEM, albeit not by IAA.

Among the peptidyl diazomethylketone inhibitors tested, only Z-Phe-Phe-CHN$_2$ and Z-Val-Val-Tyr-CHN$_2$ impaired the proteolytic degradation of vWF (cf. Fig. 9B).

Polypeptide subunits of degraded vWF and of other proteins:

Purified vWF (50 µl) was mixed with various dilutions of the protease (100 µl) which had been preincubated with 10 mM barium chloride for 5 minutes at 37°C, and the mixtures were dialyzed against 1 M urea, 5 mM Tris-HCl, pH 8.0, for 24 hours at 37°C. The resulting digests were subjected to SDS-PAGE after having been reduced with DTT. Immunodetection of the reduced vWF fragments war performed by using the APAAP kit.

The degradation of the intact vWF subunit was accompanied by the appearance of increasing amounts of two fragments with a molecular weight of 170 and 140 kD, respectively, as shown by immunoblots of reduced SDS-PAGE (cf. Fig. 10).

In parallel experiments, three other proteins were incubated

with the purified protease, i.e. 50 μl solutions each of human
fibrinogen (0.4 mg/ml), BSA (0.2 mg/ml) or calf skin collagen
(0.4 mg/ml) each incubated with 100 μl of the undiluted protease
that had been preincubated with barium ions, and the incubation
mixtures were dialyzed against 1 M urea, 5 mM Tris-HCl, pH 8.0,
for 24 hours at 37°C. Furthermore, citrated human normal plasma
(dilution 1 : 100) was dialyzed together with the protease
preparation according to the invention. In control experiments,
the protease was replaced by 0.15 M sodium chloride/0.01 M Tris-
HCl, pH 7.4. After the dialysis, the proteins were reduced with
DTT and applied to an SDS-PAGE. Coomassie-Blue staining was used
for the detection of the polypeptide chains.

It was shown that under vWF degrading conditions, no
degradation of human fibrinogen, BSA or calf skin collagen could
be observed with the protease preparation according to the
invention (cf Fig. 11). The reduced SDS-PAGE showed unchanged
subunit chains of these proteins which had been used as
potential substrates, indicating that the protease of the
invention has a high specificity for vWF.

Amino acid analysis and amino acid sequence of vWF and its
degradation products:

All three electrophoretic bands shown in Fig. 10, i.e. the
250, 170 and 140 kD bands, were transferred onto PVDF membrane
and subjected to analysis of amino acid composition and amino
acid sequence. The results are shown in Table 2 and demonstrate
good agreement between the amino acid composition of these three
polypeptide bands and the theoretical values calculated for the
intact vWF subunit, C-terminal fragment 843-2050, and N-terminal
fragment 1-842, respectively; cf. EP-0 197 592 in this respect.
The N-terminal amino acid sequence of the 250- and 140-kD bands
was Ser-Leu-Ser-X-Arg; this sequence agrees with the N-terminal
sequence of the intact vWF subunit. Analysis of the larger
degradation product with the molecular mass 170 kD yielded an N-
terminal sequence of Met-Val-Thr-Gly-Asn corresponding to amino
acid residues 843-847 in the intact vWF subunit. These data
indicate that the purified protease cleaves the peptide bond
842Tyr- 843Met.

Table 2

Amino Acid	M_r ~ 250 kD	VWF 1-2050	M_r ~ 170 kD	VWF 843-2050	M_r ~ 140 kD	VWF 1-842
Asp + Asn	173	187	102	108	77	79
Thr	118	116	70	78	39	38
Ser	131	141	78	80	55	61
Glu + Gln	249	237	152	139	104	98
Pro	139	136	85	86	54	50
Gly	152	137	117	86	76	51
Ala	110	104	67	60	50	44
Val	171	184	87	103	66	81
Ile	75	78	41	44	30	34
Leu	163	156	86	83	73	73
Tyr	48	49	18	22	21	27
Phe	58	56	33	32	25	24
His	53	52	28	31	19	21
Lys	87	88	45	47	39	41
Arg	95	101	49	58	38	43

Proteolytic fragmentation of recombinant vWF:

Recombinant vWF factor (r-vWF), produced according to FEBS-
Letters 375, 259-262 (1995) with a concentration of 104 U
(Ag)/ml in 20 mM TBS buffer, pH 8.3, was incubated as described
above with the vWF protease preparation according to the
invention. A loss of the highest multimers was already found
after 3 h, with a formation of satellite bands similar to those
of plasmatic vWF. After 20 hours, oligomers and multimers could
no longer be detected (cf. Fig. 12A).

After copper chelate affinity chromatography, the
preparation prepared according to the invention with the vWF-
specific protease still exhibited a clearly measurable plasmin
activity (0.2 U/ml; determined by preheating 50 µl sample or
standard (human plasmin from Chromogenics, with 19.3 U/ml in 20
mM TBS buffer, pH 8.3) for one minute at 37°C and admixing with
200 µl chromogenic substrate PL1 from Immuno AG (D-
cyclohexylglycyl-L-alanyl-L-arginine-p-nitroanilide, 1 mM in TBS
buffer, pH 8.3), and subsequently measuring the kinetics of the
liberation of p-nitroaniline photometrically at 405 nm at 37°C).
Thus, it was necessary to exclude the possibility that the
protease preparation might effect a degradation of the von
Willebrand factor multimers due to plasmin present in the
preparation rather than as a result of the vWF-specific
protease.

Figs. 12B and 12C show that recombinant vWF is degraded
identically when incubated with the protease according to the
invention in the presence of a plasmin inhibitor (aprotinin) and
a protease inhibitor with less specificity for plasmin, such as
PPACK. The concentration of aprotinin and PPACK was chosen such
that no protease activity could be detected with a chromogenic
substrate of plasmin.

When incubating cryoprecipitate with vWF protease (Fig.
13A), a loss of multimers could be observed over time just as
with recombinant vWF. The satellite bands showed an increase
already after a short incubation. The degradation could not be
prevented by the addition of the protease inhibitors (aprotinin,
Fig. 13B, and PPACK, Fig. 13C); however, it could be slowed
down. If a preparation of the highest molecular weight vWF
multimers (cF. Fig. 14A) produced for this purpose was used

instead of the cryoprecipitate [laboratory preparation 10 U
(Ag)/ml in 20 mM TBS buffer, pH 8.3], a clear shift towards
lower molecular weight multimers with an increase in the
satellite bands could be detected with the vWF protease
according to the invention. Overnight digestion with plasmin
completely degraded both plasmatic vWF and recombinant vWF (cf.
Figs. 14B and 14D). In this experiment, the degradation products
of the recombinant vWF with the protease according to the
invention were the satellite bands developed and the central
bands. Actually all bands were very similar to plasmatic vWF
(Fig. 14C).

Incubation of r-vWF with plasmin (cf. Fig. 15; plasmin being
diluted to 0.2 U/ml in 20 mM TBS buffer, pH 8.3 and admixed 1:1
with the respective r-vWF preparation and incubated at 37°C) at
lower concentration, based on the substrate present, showed a
successive degradation of the multimers over 29 h (cf. Fig. 15A)
in the kinetic examination. A control preparation without
protease demonstrates the otherwise found stability of the r-vWF
in solution (cF. Fig. 15C). When reducing the plasmin
concentration to 1 : 10, r-vWF was degraded more slowly (Fig.
15B); this could be prevented by the addition of aprotinin (cf.
Fig. 16B) and PPACK (cf. Fig. 16C). The multimers showed no
change over time (about 20 hours), in contrast to the situation
after treatment without inhibitor (cf. Fig. 16A).

The control experiment with cryoprecipitate (Fig. 17) with
incubation for more than 8 hours again shows a reduction of the
high multimers which could be prevented by adding aprotinin or
PPACK.

Further tests investigated whether a proteolytic degradation
of r-vWF took place during incubation with human plasma (cf.
Fig. 18). For this, the r-vWF preparation was mixed with equal
volumes of a vWF-deficient plasma (cf. Fig. 18A) and incubated
for 48 hours, samples being taken at various times. No change of
the r-vWF over time could be detected. A control preparation
allowed to stand for an equal length of time at 37°C in buffer
media also showed no change of structure (cf. Fig. 8B).

Analysis of the r-vWF and its proteolytic degradation
products was performed on 1% and 2% agarose gels, also according
to the method of Ruggeri et al. (Blood 57 (1981), 1140-1143).

The vWF multimers were made visible by immunoenzymatic staining
according to Aihara et al. (Thromb. Haemostas. 55 (1986), 263-
267). A rabbit-anti-von Willebrand factor-antiserum (from
Dakopatts, Glostrup, Denmark) was used as the primary antibody,
and an alkaline phosphatase-conjugated, affinity-purified goat-
anti-rabbit IgG H+L-antibody (from Axell Accurate Chemical and
Scientific Corp, Westburg, N.Y.) served as the secondary
antibody. Staining of the protein bands was effected by means of
the nitroblue tetrazolium/bromochloro-indolyl-phosphate
substrate system.

C l a i m s :

1. A purified multimerase comprising an indirect or a direct
proteolytic activity and converting von Willebrand factor (vWF)
having a singlet structure to vWF having a satellite structure,
said purified multimerase being active in the presence of the
serine protease inhibitor DFP or the calpain protease inhibitor
Z-Leu-Leu-Tyr-CHN$_2$.

2.   A multimerase as set forth in claim 1 capable of cleaving
the peptide bond 842Tyr-843Met of vWF.

3.   A multimerase as set forth in claim 1, capable of being
detected in a fraction obtained after gel filtration of one of a
plasma and serum fraction corresponding to a molecular weight of
more than 200 kD.

4.   A multimerase as set forth in claim 1 and present in an at
least 1,000-fold enriched form as compared to plasma.

5.   A multimerase as set forth in claim 1 comprising a specific
activity of at least 10 U/mg protein in the presence of a serine
protease inhibitor.

6.   A multimerase as set forth in claim 1 which has a pH
optimum in the range of from 7 to 10.

7.   A multimerase as set forth in claim 1 which is active in
the presence of bivalent metal ions.

8.   A multimerase as set forth in claim 7, wherein said metal
ions are alkaline earth ions.

9.   A multimerase as set forth in claim 1 which has an activity
capable of being increased by shearing forces, reduced ionic
strength or chaotropic agents.

10.   A multimerase as set forth in claim 1 which has an
enhanced activity relative to modified vWF.

11.   A multimerase as set forth in claim 10, wherein said
modified vWF is partially denatured vWF.

12.   A multimerase as set forth in claim 10, wherein said
modified vWF is a vWF having a conformational change.

13.   A method of purifying a multimerase having an indirect or
a direct proteolytic activity and converting von Willebrand
factor (vWF) of singlet structure to vWF of satellite structure,
said purified multimerase being active in the presence of the
serine protease inhibitor DFP or the calpain protease inhibitor
Z-Leu-Leu-Tyr-CHN$_2$, from plasma, serum, a plasma fraction or a
serum fraction by a chromatographic method, said method
comprising recovering said purified multimerase from those
fractions in which the proteolytic activity for inactivating vWF
is found in the presence of a serine protease inhibitor.

14.   A method as set forth in claim 13, wherein said
multimerase is recovered by ion exchange chromatography,
affinity chromatography or gel filtration.

15.   A method of producing a vWF of satellite structure
comprising incubating a vWF of singlet structure with a purified
multimerase comprising an indirect or a direct proteolytic
activity and converting von Willebrand factor (vWF) having a
singlet structure to vWF having a satellite structure, said
purified multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, so as to obtain a vWF of satellite structure, and
recovering said vWF of said satellite structure.

16.   A method as set forth in claim 15, wherein said incubating
is performed in vitro.

17.   A method as set forth in claim 15, wherein said incubating
is performed in vivo.

18.   A method as set forth in claim 15, wherein said incubating
is performed ex vivo.

19.   A preparation comprising a multimerase activity derived
from a purified multimerase with an indirect or a direct
proteolytic activity and converting von Willebrand factor (vWF)
of singlet structure to vWF of satellite structure, said
purified multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, said purified multimerase further being present in
an at least 1,000-fold enriched form as compared to plasma.

20.   A pharmaceutical composition comprising a multimerase
having an indirect or a direct proteolytic activity and
converting von Willebrand factor (vWF) of singlet structure to
vWF of satellite structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, which has been treated for an inactivation or
removal of viruses.

21.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase has an optimum proteolytic activity at
a pH in a range of from 7 to 10.

22.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase is provided in a fraction obtained
after gel filtration of a plasma fraction or a serum fraction
corresponding to a molecular weight of more than 200 kD.

23.   A pharmaceutical composition as set forth in claim 22,
wherein said molecular weight is approximately 300 kD.

24.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase is enriched by at least 1,000 times as
compared to plasma.

25.   A pharmaceutical composition as set forth in claim 20;

wherein said multimerase is enriched by at least 10,000 times as
compared to plasma.

26.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase has a specific activity of at least 10
U/mg protein in the presence of a serine protease inhibitor.

27.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase is active in the presence of alkaline
earth ions.

28.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase has an activity capable of being
increased by shearing forces, reduced ionic strength or
chaotropic substances.

29.   A pharmaceutical composition as set forth in claim 20,
wherein said multimerase has an increased activity relative to
modified vWF.

30.   A pharmaceutical composition as set forth in claim 29,
wherein said modified vWF is partially denatured vWF.

31.   A pharmaceutical composition as set forth in claim 29,
wherein said modified vWF is vWF having a conformational change.

32.   A pharmaceutical composition as set forth in claim 20,
wherein said treatment of said multimerase for inactivating
viruses is at least a treatment with tensides, a heat treatment
or combinations thereof.

33.   A pharmaceutical composition as set forth in claim 20,
wherein said treatment for removal of viruses is a
nanofiltration.

34.   A method for treating and preventing thromboses and
thromboembolic disorders in patients, comprising administering
to said patients an effective dose of a multimerase having an
indirect or a direct proteolytic activity and converting vWF of

- 33 -

singlet structure to vWF of satellite structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, wherein said multimerase has been treated for an
inactivation or removal of viruses.

35.   A method for treating and preventing a supranormal vWF
content or an increased level of high- molecular vWF in
patients, comprising administering to said patients an effective
dose of a multimerase having an indirect or a direct proteolytic
activity and converting vWF of singlet structure to vWF of
satellite structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, wherein said multimerase has been treated for an
inactivation and removal of viruses.

36.   A method for treating and preventing a supranormal vWF
antigen or supranormal vWF activity in patients, comprising
administering to said patients an effective dose of a
multimerase having an indirect or a direct proteolytic activity
and converting vWF of singlet structure to vWF of satellite
structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, wherein said multimerase has been treated for an
inactivation and depletion of viruses.

37.   A method as set forth in claim 36 for treating and
preventing the primary hemostatic activity as well as the
binding activity to the subendothelium, to thrombocytes, to
thrombocyte adhesion proteins, such as GPIb and GPIIb/IIIa, to
collagen, to factor VIII and to heparin.

38.   A method for treating and preventing thrombotic
thrombocytopenic purpura, Henoch-Schönlein purpura,
preeclampsia, neonatal thrombocytopenia or hemolytic- uremic
syndrome in patients, comprising administering to said patients
an effective dose of a multimerase having an indirect or a

direct proteolytic activity and converting vWF of singlet structure to vWF of satellite structure,
said multimerase being active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, wherein said multimerase has been treated for an inactivation and removal of viruses.

39.   A method of producing a pharmaceutical preparation comprising
-providing a multimerase-containing starting material, said multimerase having an indirect or a direct proteolytic activity and converting vWF of singlet structure to vWF of satellite structure, said multimerase being active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$,
-purifying said multimerase-containing starting material by chromatographic methods, and
-subsequently formulating said chromatographically purified multimerase-containing material to a pharmaceutical preparation by known methods.

40.   A method as set forth in claim 39, wherein said multimerase-containing starting material is produced by a suitable expression system.

41.   A method of determining the proteolytic activity of a multimerase having an indirect or a direct proteolytic activity and converting vWF of singlet structure to vWF of satellite structure,
said multimerase being active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, said method comprising
    - incubating a fraction containing said multimerase with a vWF having singlet structure, and
    - determining the reaction rate of said vWF.

42.   A method of determining the proteolytic activity of a multimerase having an indirect or a direct proteolytic activity and converting vWF of singlet structure to vWF of satellite

structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, said method comprising

- incubating a fraction containing said multimerase with a
  biologically active vWF in the presence of a serine protease
  inhibitor, and
- determining the extent of inactivation of said vWF.

43.   A method of determining the proteolytic activity of a
multimerase having an indirect or a direct proteolytic activity
and converting vWF of singlet structure to vWF of satellite
structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, said method comprising

- incubating a fraction containing said multimerase with a
  chromogenic substrate which is homologous to the vWF
  sequence containing the peptide bond 842Tyr-843Met and
  includes a chromogenic group,
- thereby cleaving said substrate by proteolytic activity so
  as to form a chromophore, and
- determining the colour development.

44.   A method of preparing antibodies against a multimerase
having an indirect or a direct proteolytic activity and
converting vWF of singlet structure to vWF of satellite
structure,
said multimerase being active in the presence of the serine
protease inhibitor DFP or the calpain protease inhibitor Z-Leu-
Leu-Tyr-CHN$_2$, comprising using said multimerase in purified form
as an immunogen.

45.   An antibody preparation comprising antibodies directed
against a multimerase having an indirect or a direct proteolytic
activity and converting vWF of singlet structure to vWF of
satellite structure,
said multimerase being active in the presence of the serine

protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$.

46. An antibody prepration as set forth in claim 45, further comprising a solid phase, said antibodies being immobilized on said solid phase.

47. A method for detecting or purifying a multimerase having an indirect or a direct proteolytic activity and converting vWF of singlet structure to vWF of satellite structure, said multimerase being active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, wherein said multimerase has been treated for an inactivation and depletion of viruses, said method comprising the binding of said multimerase to antibodies capable of binding the multimerase and subsequent separating said multimerase from said antibodies.

48. A storage-stable pharmaceutical preparation comprising a vWF having a permanent multimer pattern.

49. A preparation as set forth in claim 48, further comprising factor VIII, a factor VIII derivative or a factor VIII mutant.

50. A preparation as set forth in claim 48, wherein said vWF is a native vWF, rvWF, a vWF derivative, a vWF fragment or a vWF mutant, and the vWF contains the Tyr842-Met843 peptide sequence.

51. A preparation as set forth in claim 48, comprising a reduced multimerase activity.

52. A preparation as set forth in claim 51, wherein said multimerase activity is reduced to a content of less than the detection limit.

53. A preparation as set forth in claim 51, wherein said multimerase activity is reduced by inhibition of said multimerase.

54.   A preparation as set forth in claim 51, wherein said multimerase activity is reduced by depletion of said multimerase.

55.   A preparation as set forth in claim 48 which is storage-stable in the liquid state.

56.   A preparation as set forth in claim 48 which is storage-stable in the liquid-deepfrozen state.

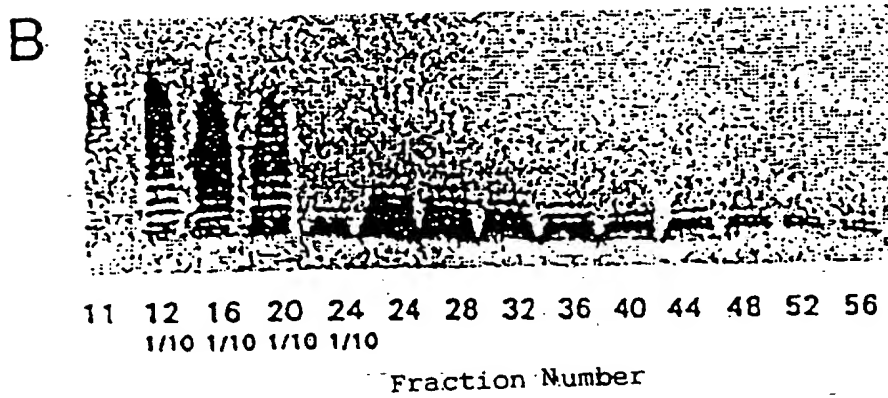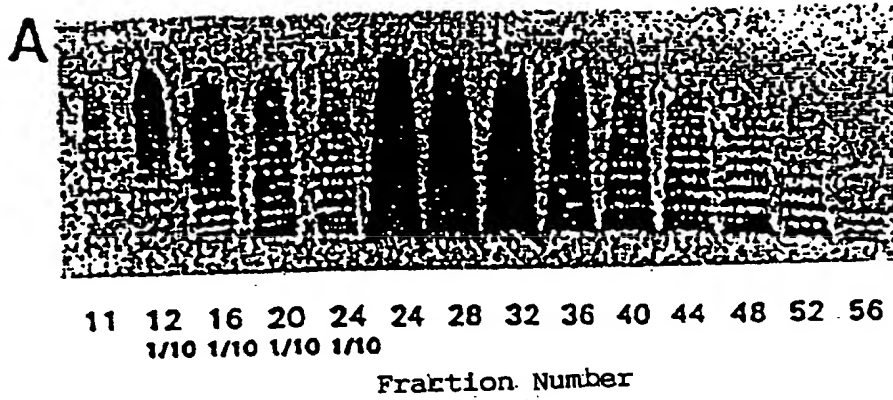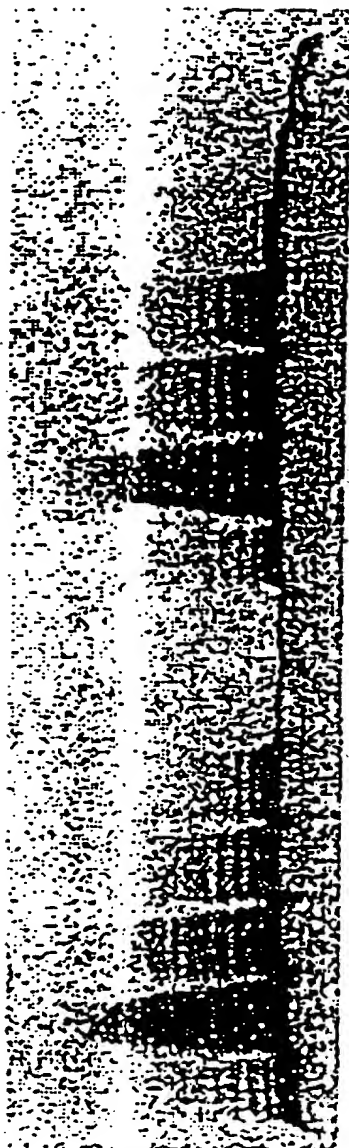57.   A preparation as set forth in claim 48 which is storage-stable in the lyophilized state.

FIG. 1

FIG. 2

A

11 12 16 20 24 24 28 32 36 40 44 48 52 56
    1/10 1/10 1/10 1/10
                    Fraction Number

B

11 12 16 20 24 24 28 32 36 40 44 48 52 56
    1/10 1/10 1/10 1/10
                    Fraction Number

FIG. 3
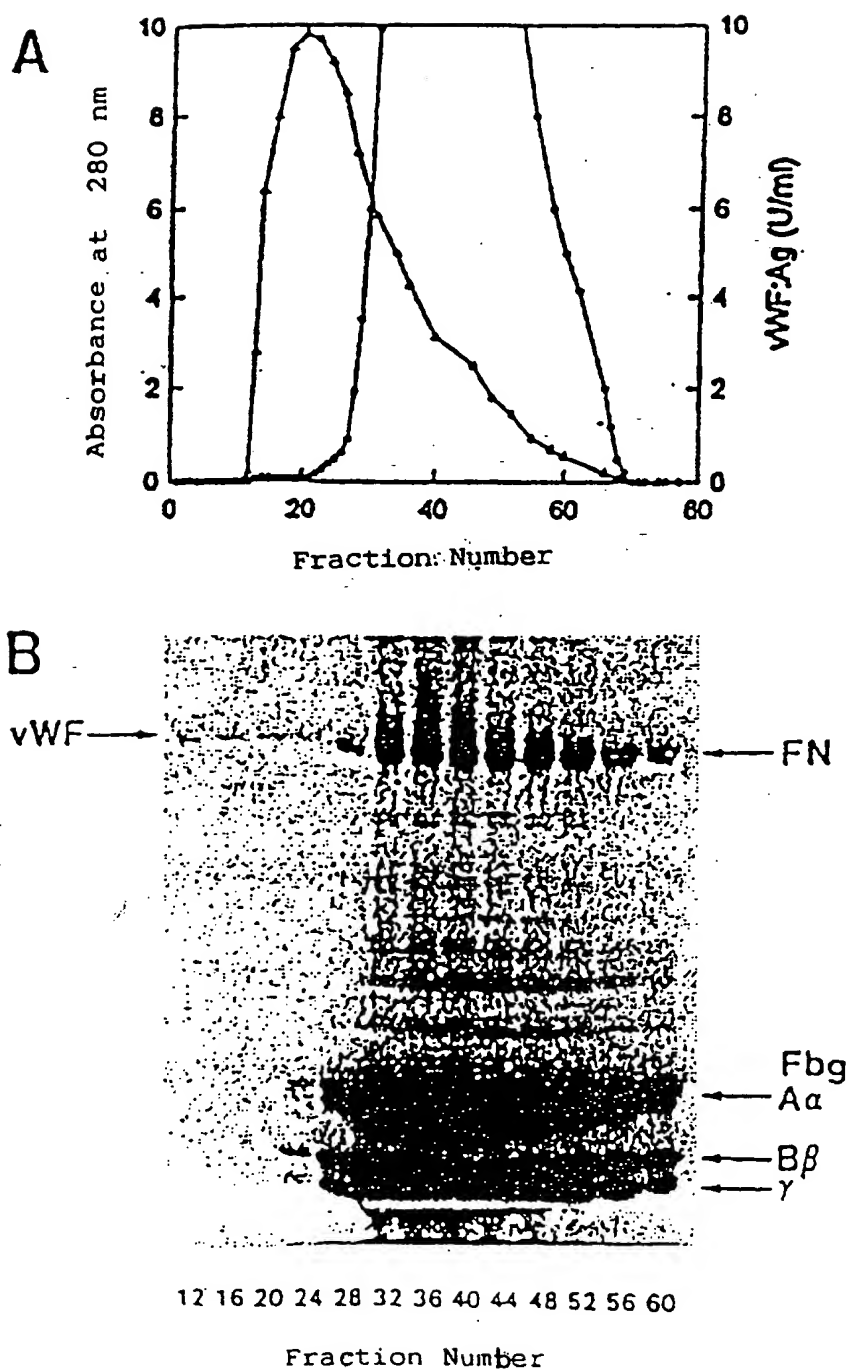
NaCl (M)	0	0.16	0.16	0.10	0.05	0.015	0	0.16	0.10	0.05	0.015	0	0	0.15	0.15	0.10	0.05	0.015	0
Urea (M)	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1	1	1	1	1
DFP (mM)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1

FIG. 4

Lysed Platelets (x 15): Sediment

Lysed Platelets (x 15): Supernatant

PPP

Cryoprecipitate-free PPP

Cryoprecipitate (x 15)

Defibrinated PPP

Serum

FIG. 5

6/18

FIG. 6

FIG. 7

FIG. 8

FIG. 9

A

Control
10 mM EDTA
10 mM EGTA
10 mM CITRATE
10 mM IAA
10 mM NEM
1 mM DFP
1 mM PMSF
1 mM TLCK
1 mM TPCK
0.01 mM LEUPEPTIN
0.01 mM APROTININ

B

Control
0.1 mM ZLLYCHN$_2$
0.1 mM ZVVYCHN$_2$
0.1 mM FACHN$_2$
0.1 mM ZF(I)ACHN$_2$
0.1 mM ZYACHN$_2$
0.1 mM ZFFCHN$_2$

FIG. 10

Protease Dilution

FIG. 11

24h 37˚C
Control

24h 37˚C
+ Protease

A B C D   A B C D
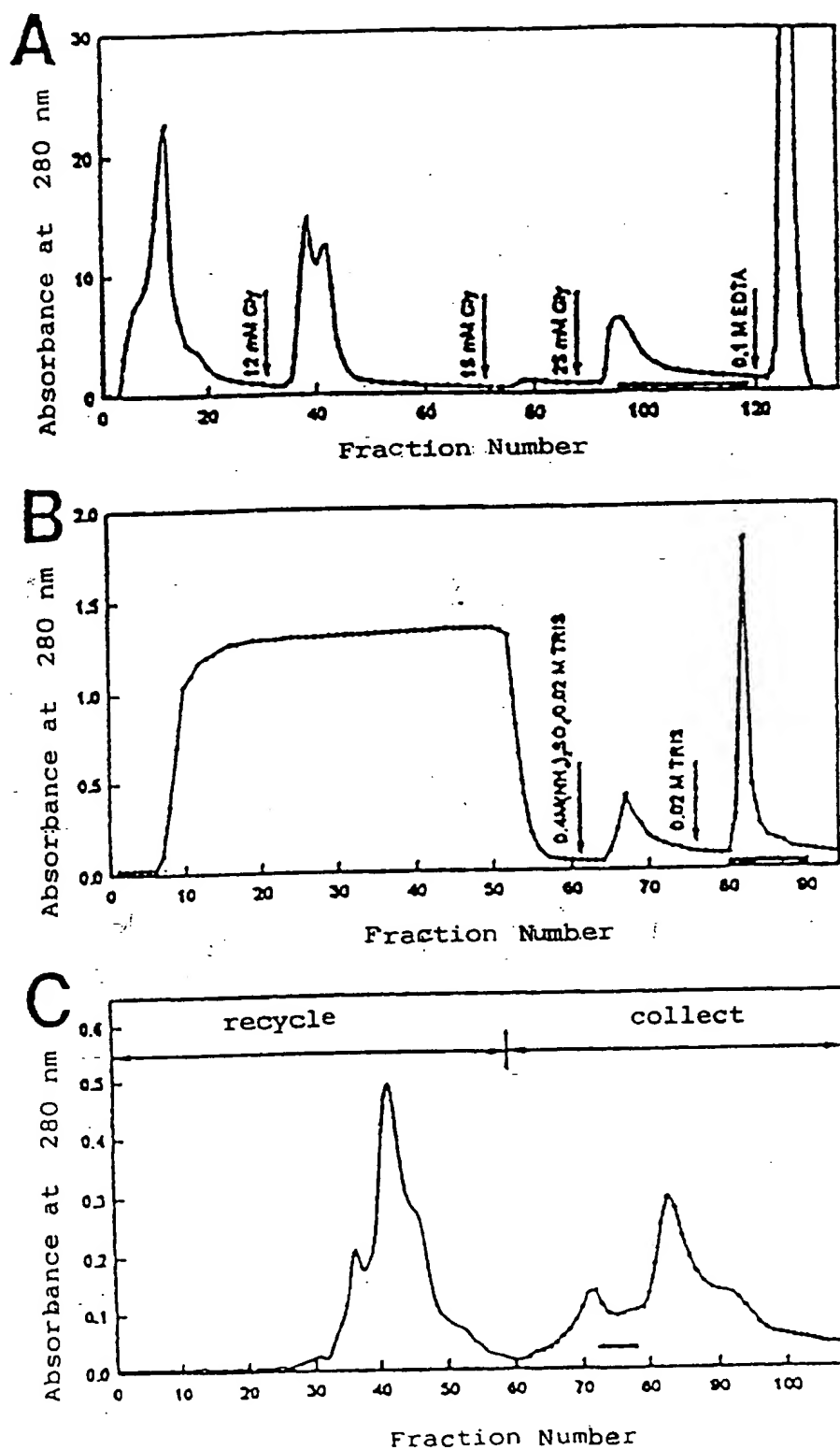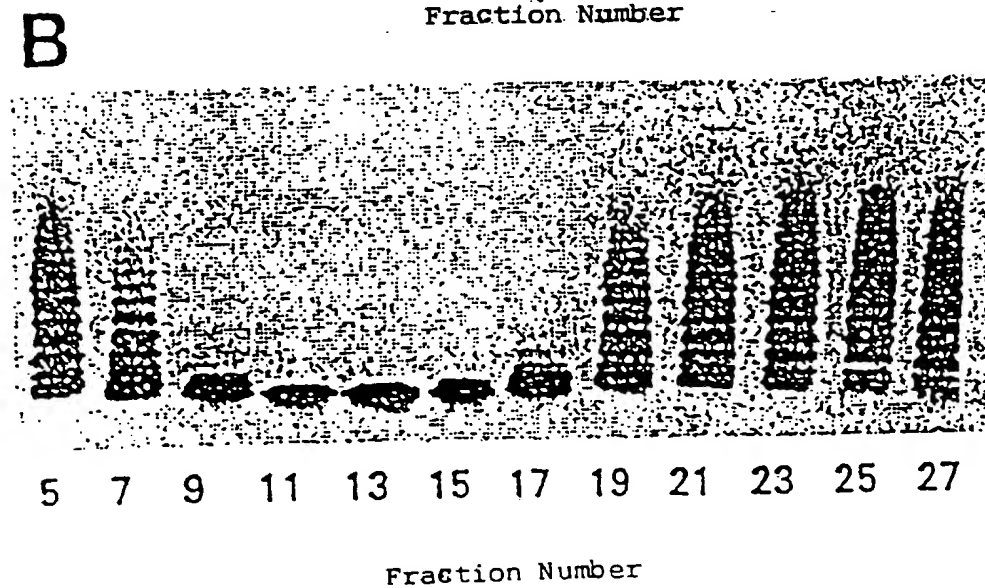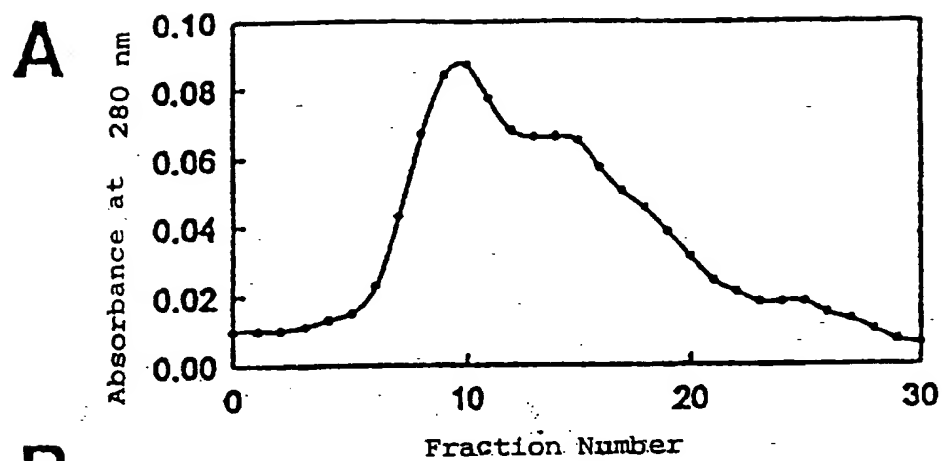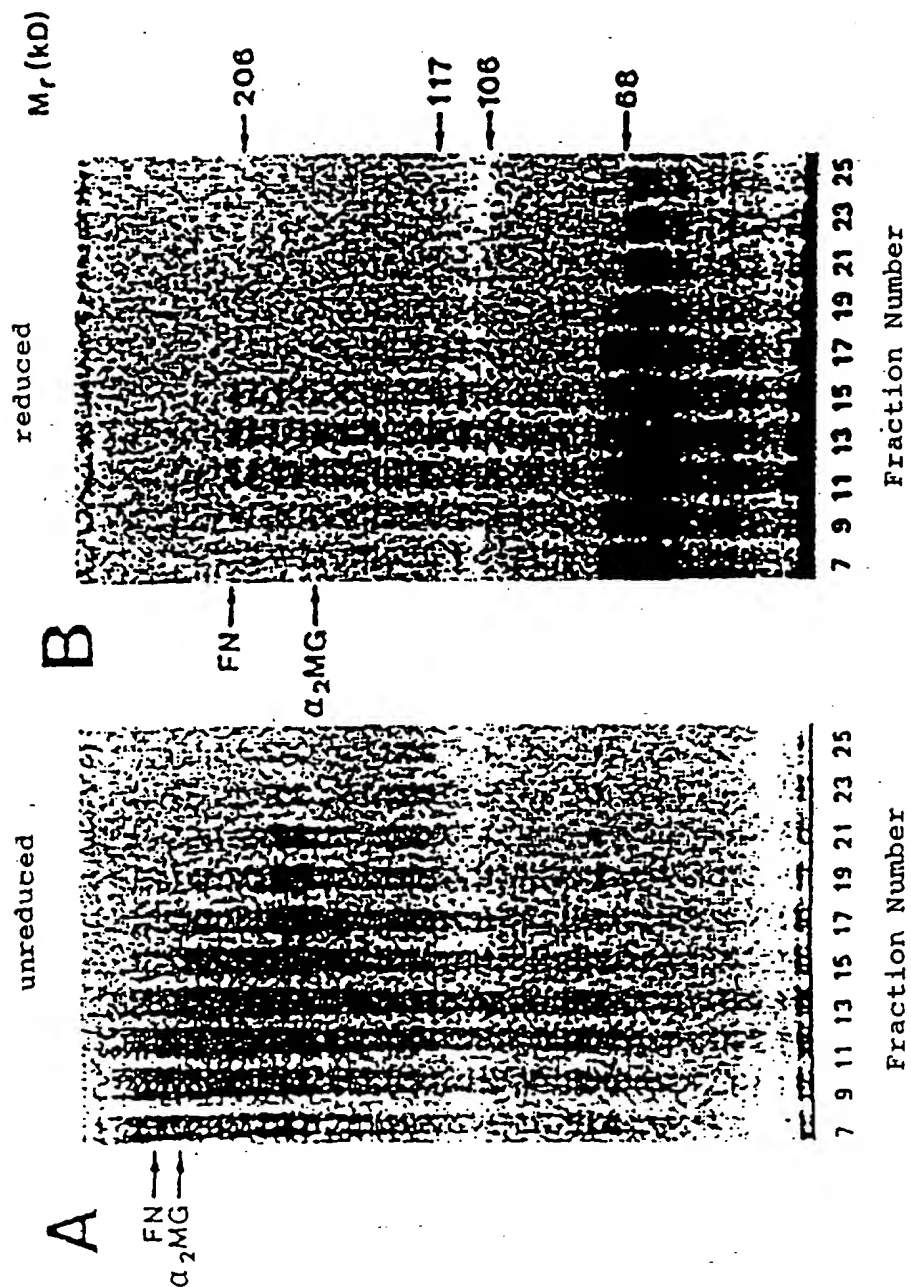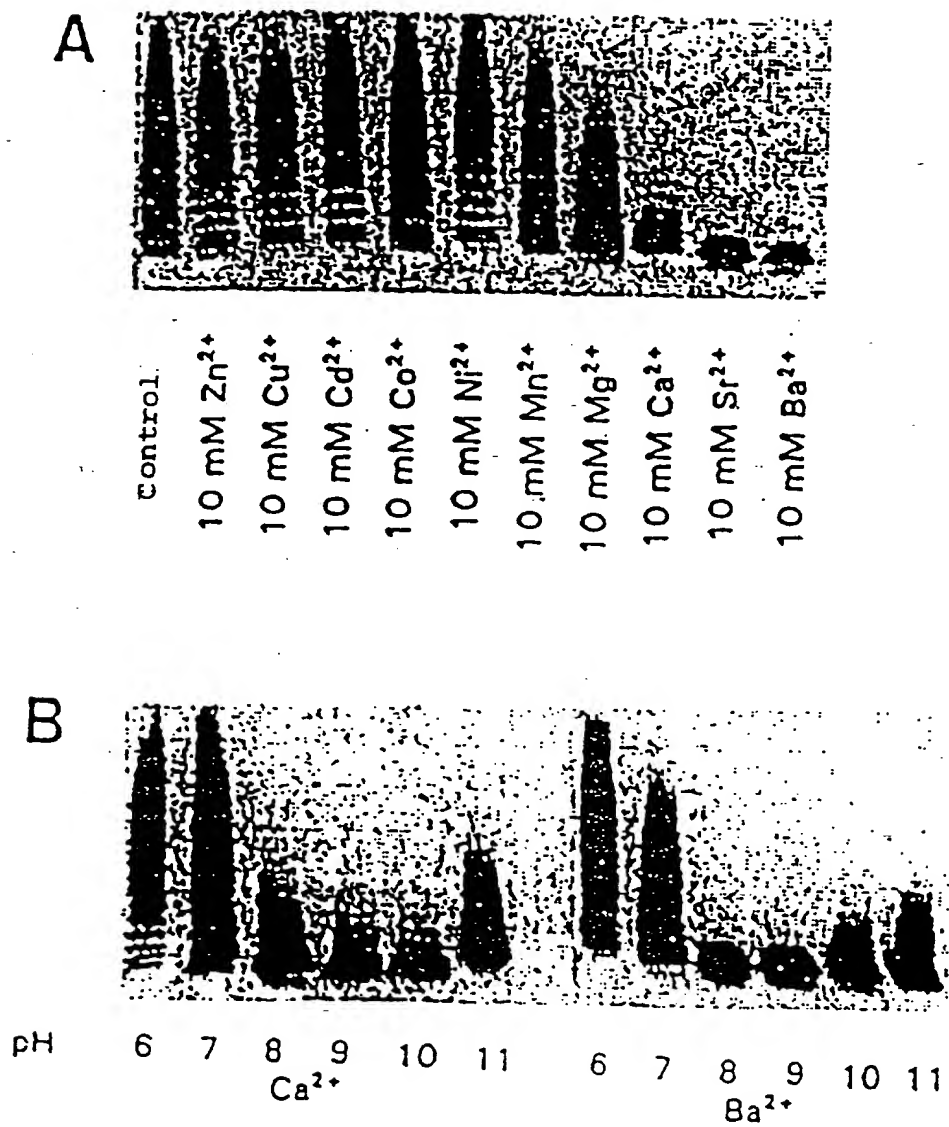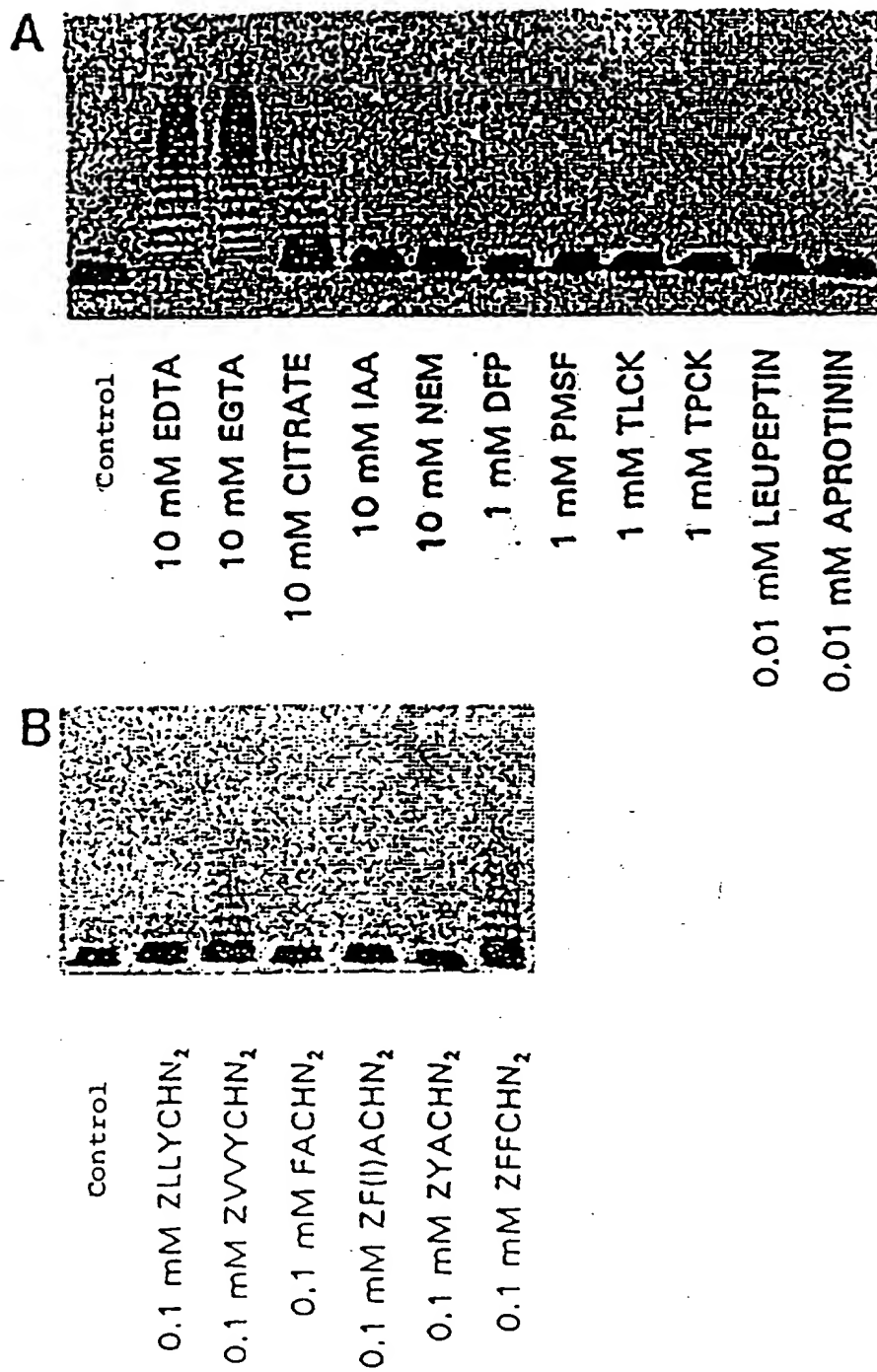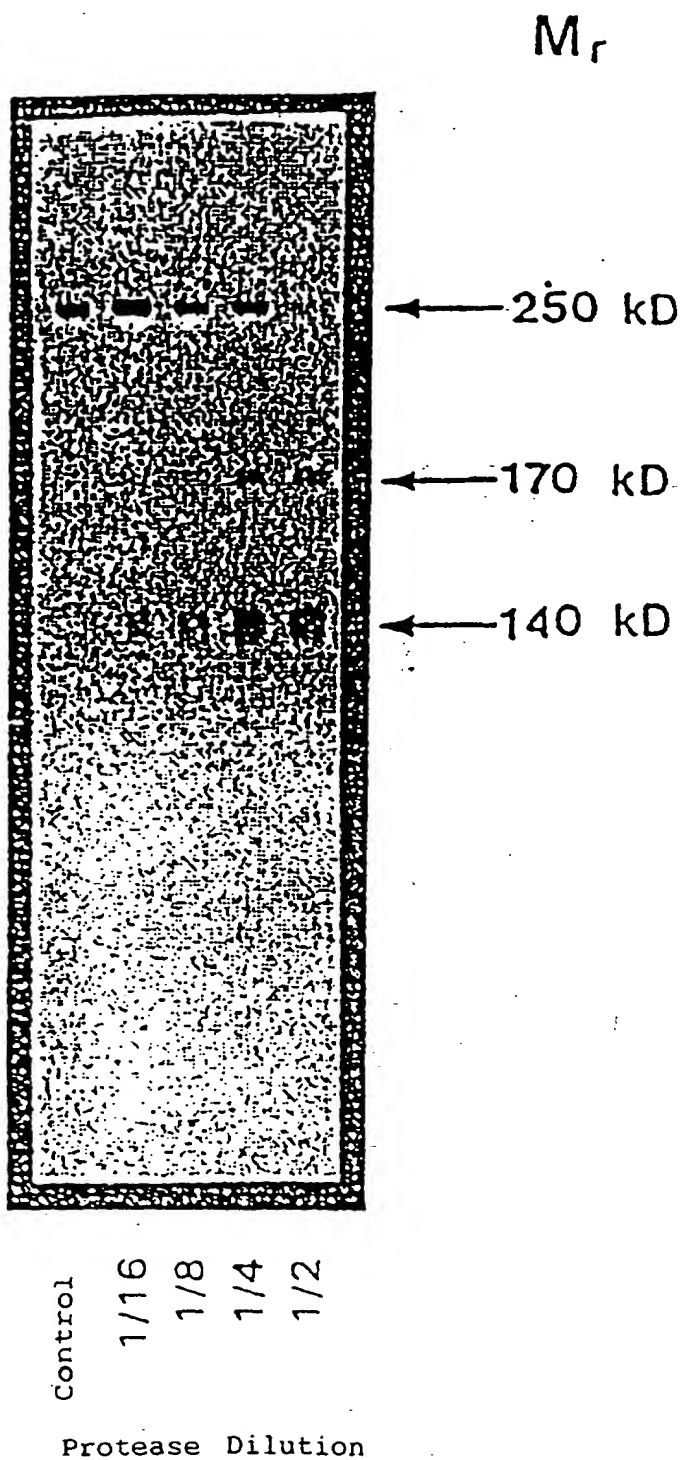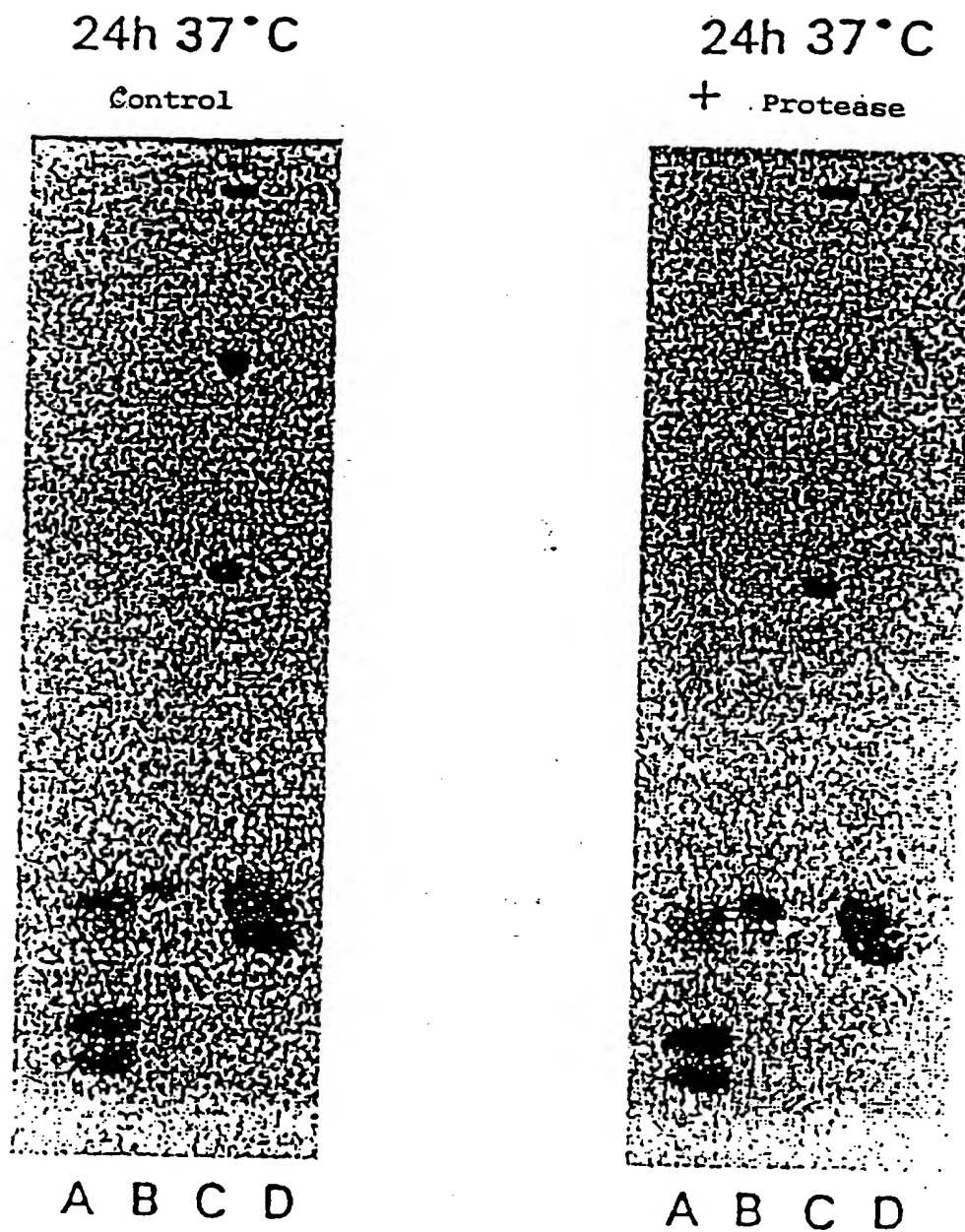
FIG. 12
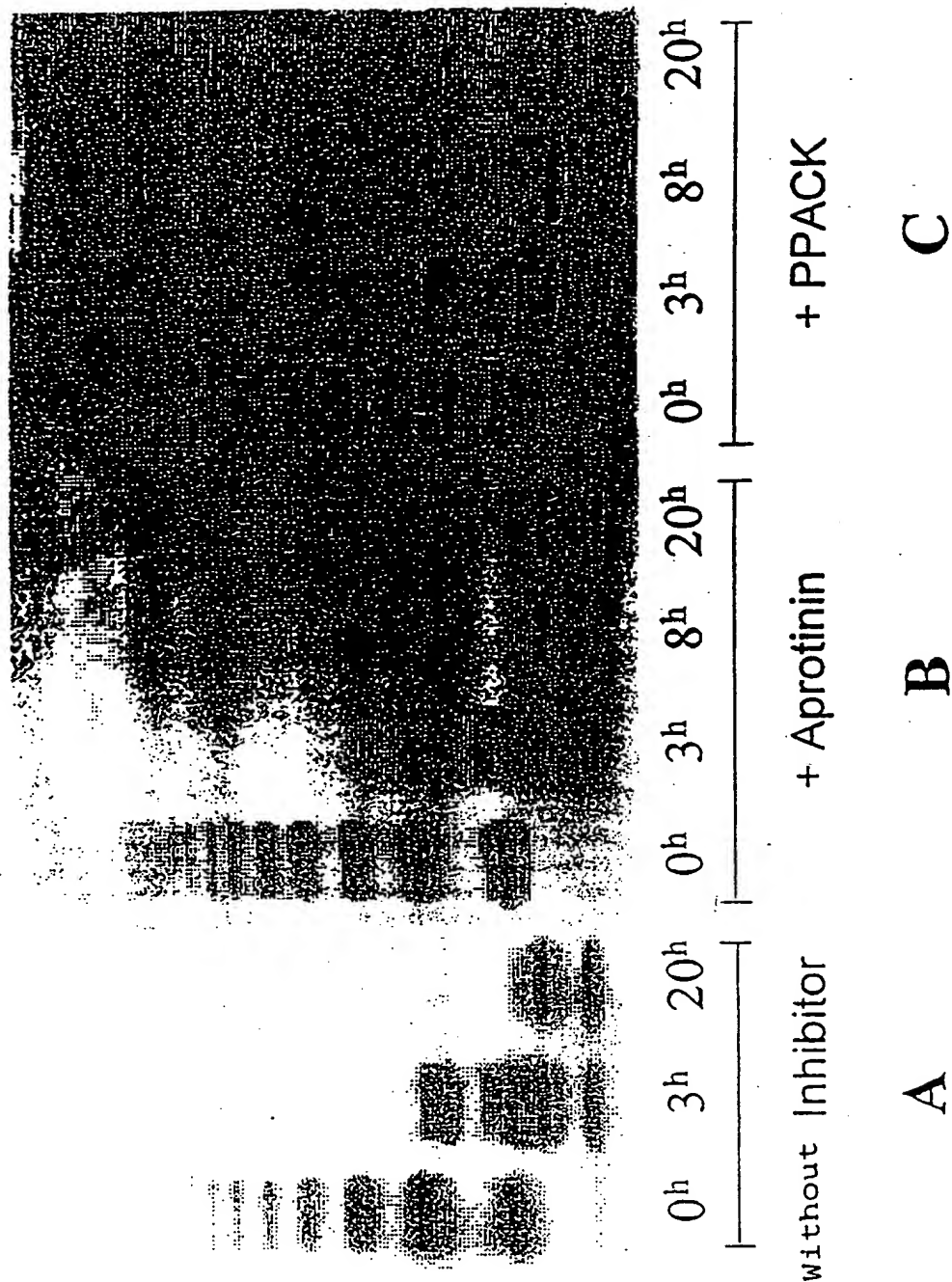
Degradation of rvWF

FIG. 13

Degradation of vWF from Cryoprecipitate
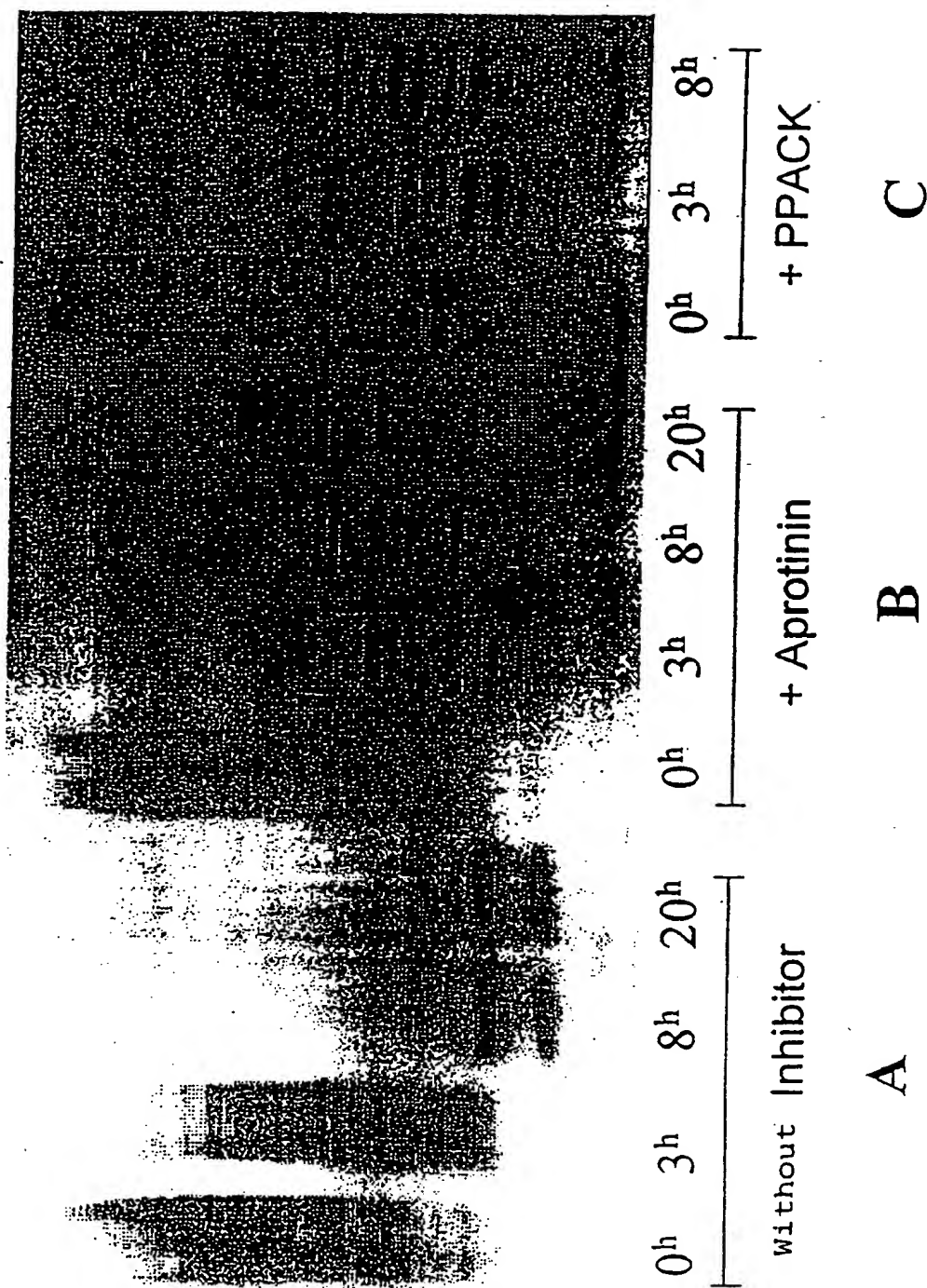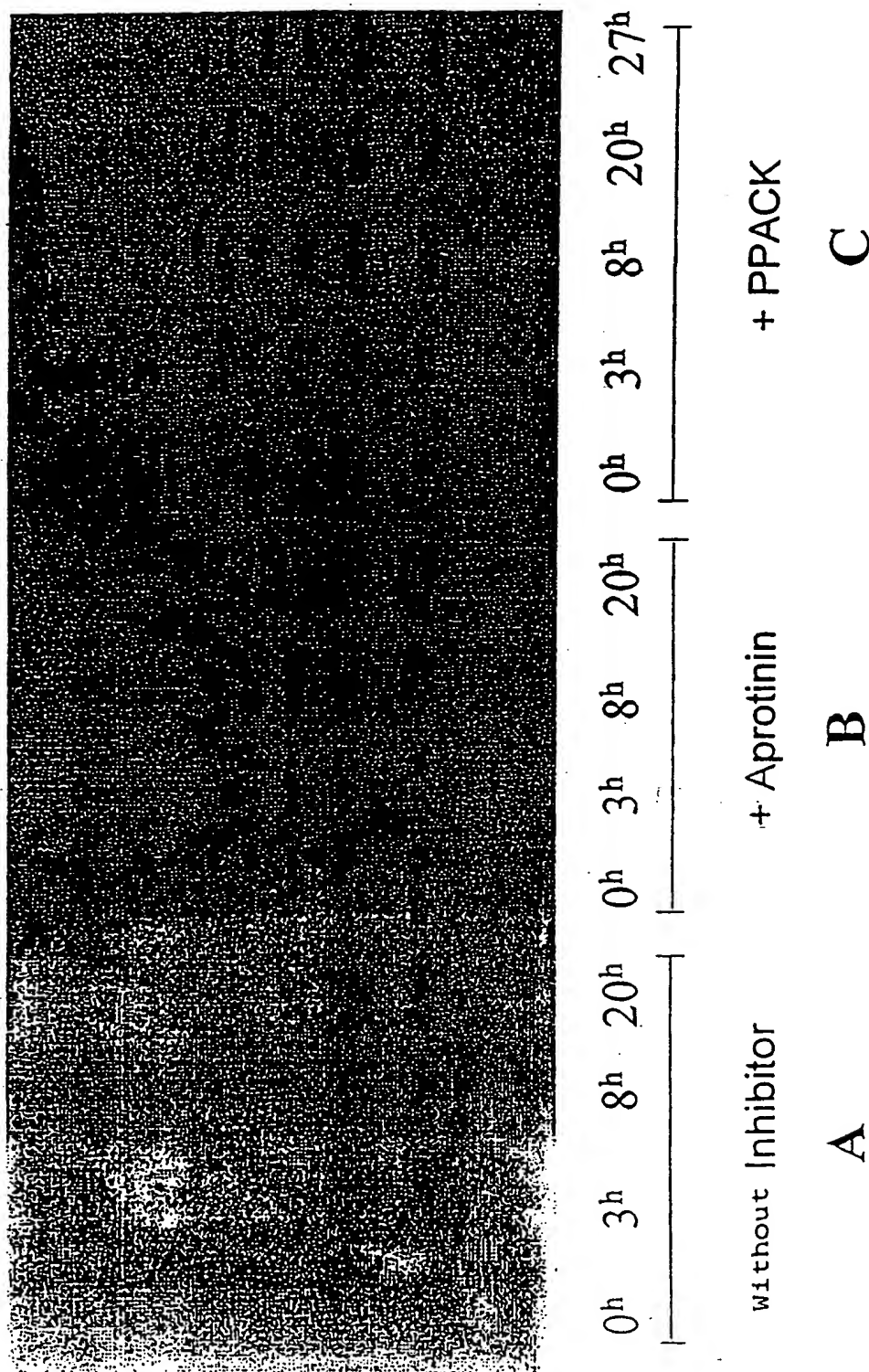
FIG. 14

Degradation of vWF-High Multimers and rvWF

vWF-
High Multimers

A   after
     "vW-Multimerase"

B   after Plasmin

rvWF

C   after
    "vW-Multimerase"

D   after Plasmin
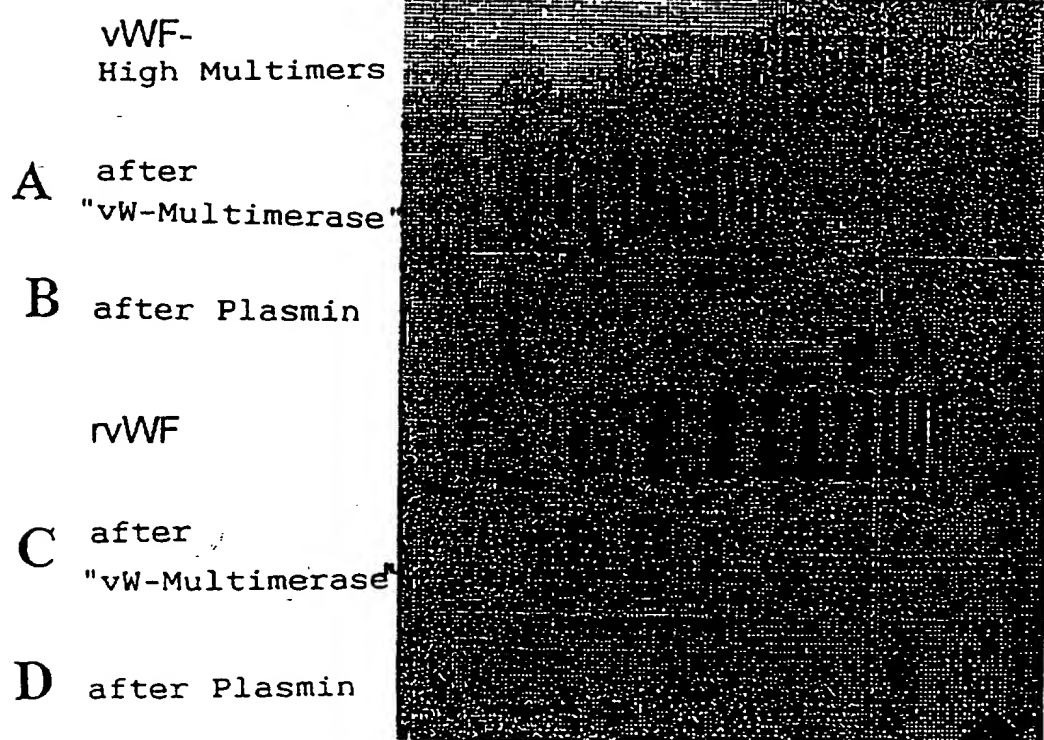
FIG. 15　Degradation of rvWF with Plasmin (Kinetics)
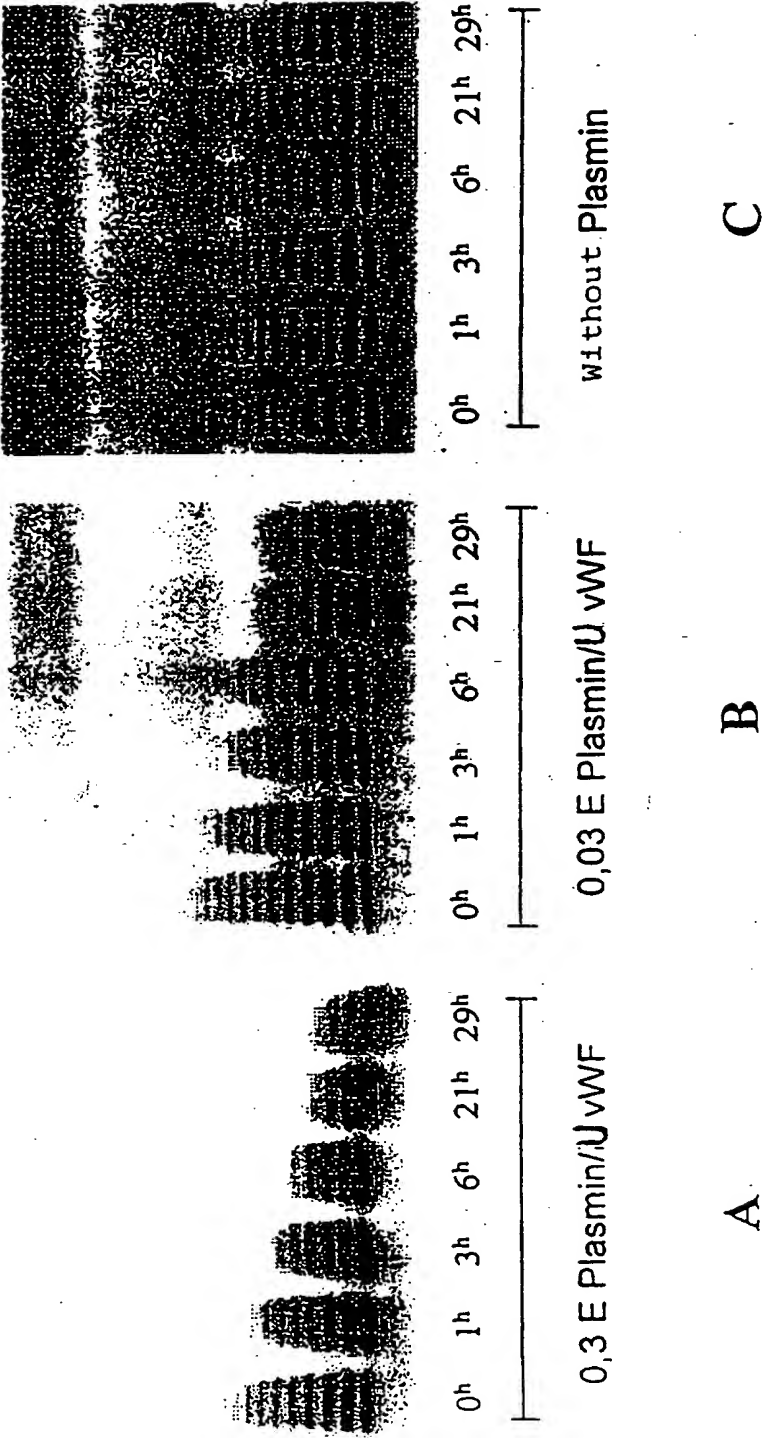
FIG. 16   Degradation of rVWF with Plasmin ± Inhibitors

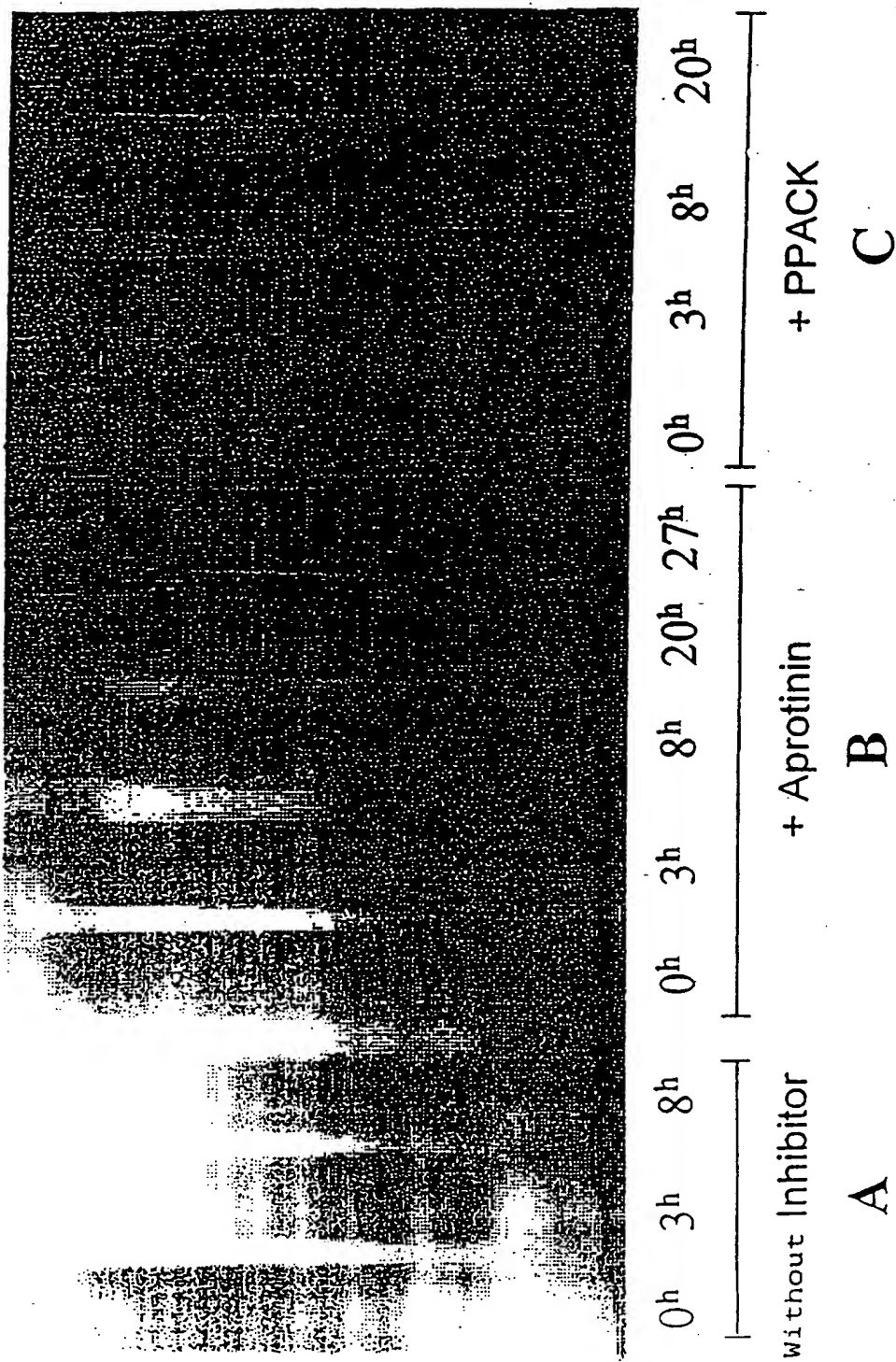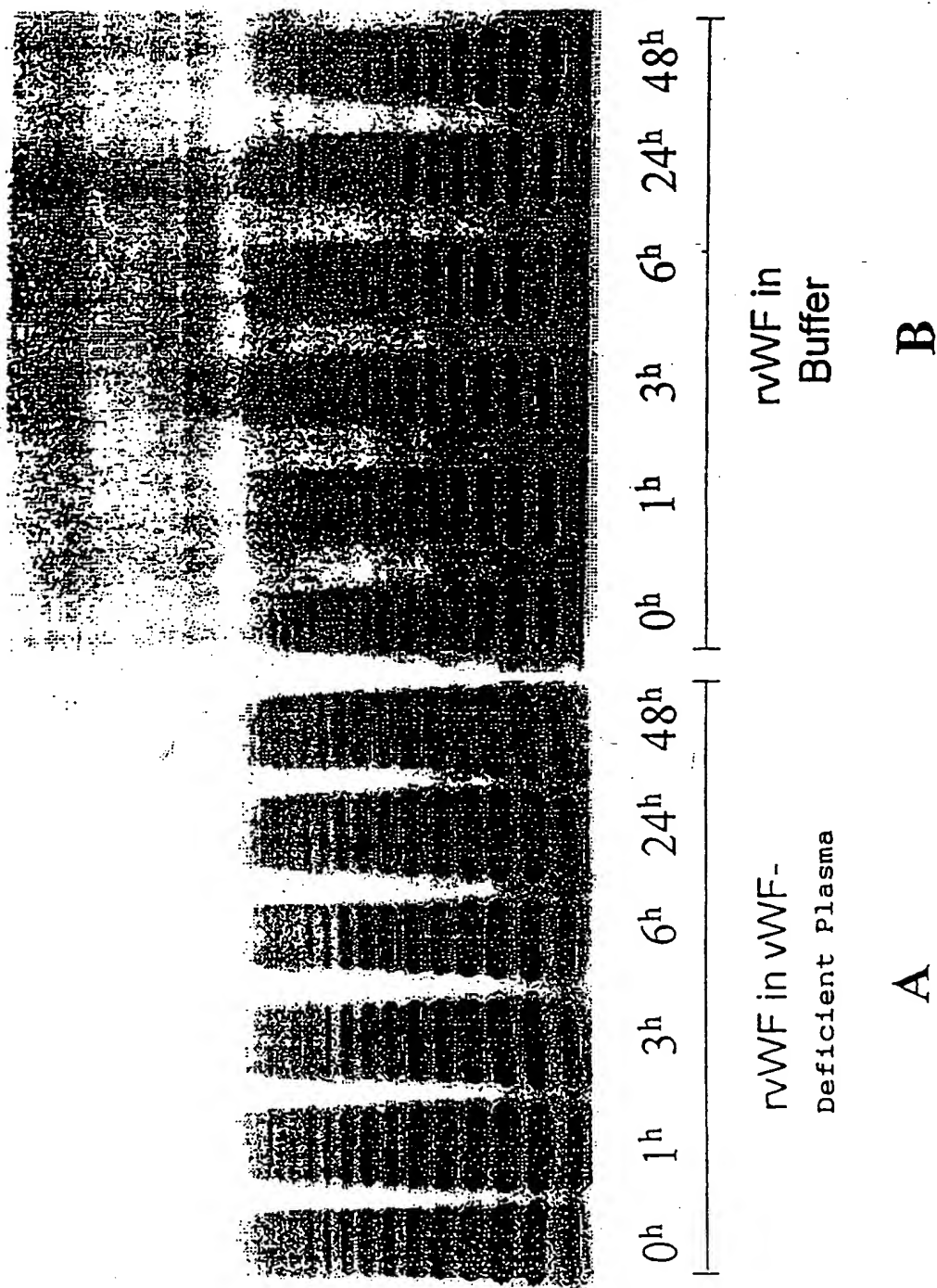FIG. 17 Degradation of vWF from Cryoprecipitate with Plasmin

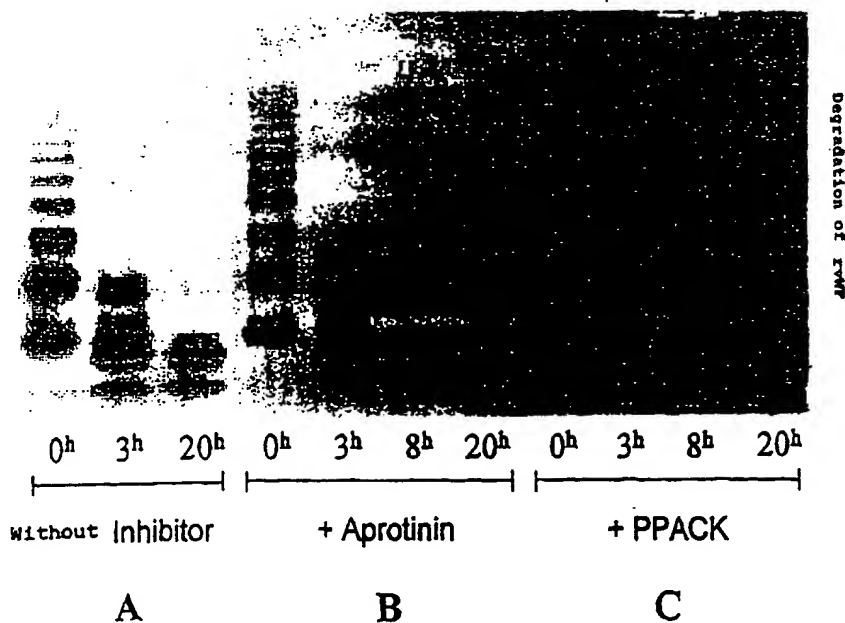FIG. 18

rvWF-Incubation in vWF-Deficient Human Plasma

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : C12N 9/64, C07K 14/755, A61K 38/48, 38/37, C12Q 1/37, C07K 16/40	A3	(11) International Publication Number: **WO 97/41206** (43) International Publication Date: 6 November 1997 (06.11.97)

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(74) Agents: SONN, Helmut et al.; Riemergasse 14, A-1010 Wien
   (AT).

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(88) Date of publication of the international search report:
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</table>

(54) Title: VON WILLEBRAND FACTOR MULTIMERASE

(57) Abstract

   There is disclosed a purified multimerase having an indirect and a direct proteolytic activity, which converts vWF having a singlet structure to vWF having a satellite structure and is active in the presence of the serine protease inhibitor DFP or the calpain protease inhibitor Z-Leu-Leu-Tyr-CHN$_2$, as well as a method of preparing the multimerase according to the invention.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N9/64   C07K14/755   A61K38/48   A61K38/37   C12Q1/37
         C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 503 991 A (CENTRE REGIONAL DE TRANSFUSION SANGUINE DE LILLE) 16 September 1992 see page 2, line 1 - line 3 see page 2, line 58 - page 3, line 18 see page 3, line 49 - page 4, line 23 ---	48-50, 55-57
A	DE 44 35 485 A (IMMUNO AG) 21 March 1996 see page 2, line 3 - line 6 see page 3, line 29 - line 36 & WO 96 10584 A cited in the application --- -/--	48-57

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1997	23.10.97

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Fax: (+31-70) 340-3016 | Authorized officer

MONTERO LOPEZ B. |

1

INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/EP 97/02131

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THOMAS J. KUNICKI ET AL.: "Cleavage of human von Willebrand factor by platelet Calcium-activated protease" BLOOD, vol. 65, no. 2, February 1985, pages 352-356, XP002042008 see abstract see page 355, right-hand column, paragraph 2 - page 356, right-hand column, paragraph 1	1-47
A	JUDITH A. DENT ET AL.: "Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, August 1990, WASHINGTON US, page 6306-6310 XP002042009 see abstract see page 6309, left-hand column, paragraph 3 - right-hand column, paragraph 3	1-47
A	BERKOWITZ, SCOTT D. ET AL: "Sialic acid prevents loss of large von Willebrand factor multimers by protecting against amino-terminal proteolytic cleavage" BLOOD (1988), 72(5), 1790-6 CODEN: BLOOAW;ISSN: 0006-4971, 1988, XP002042010 see the whole document	1-47
P,X	FURLAN, MIHA ET AL: "Partial purification and characterization of a protease from human plasma which cleaves von Willebrand factor to fragments produced by in vivo proteolysis" BLOOD (1996), 87(10), 4223-4234 CODEN: BLOOAW;ISSN: 0006-4971, 1996, XP002042011 see abstract see page 4227, left-hand column, paragraph 2 - page 4233, left-hand column, paragraph 1	1-8, 13-27

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

page 2 of 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/02131

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Remark : Although claims 34-38 are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 503991 A	16-09-92	FR 2673632 A	11-09-92
		AU 645172 B	06-01-94
		AU 1113192 A	10-09-92
		BE 1004178 A	06-10-92
		CA 2062340 A	09-09-92
		CS 9200568 A	16-09-92
		IL 101043 A	18-02-97
		IT 1256692 B	12-12-95
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		LT 266 A,B	15-07-94
		NO 301278 B	06-10-97
		NZ 241701 A	26-10-94
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DE 4435485 A	21-03-96	AU 3803195 A	26-04-96
		WO 9610584 A	11-04-96
		EP 0784632 A	23-07-97

P001   04/18/90   Original claims
P001   01/13/92   Amendment . All claims canceled; new claims 151-209
P001C   755 patent issued claims 1-59
P001C2   09/06/94   Preliminary Amendment 151-195
P001C2   327 patent issued claims 1-27
P006D No new claims
P007D 6/28/93   Preliminary Amendment claims 151- 165
P007D 05/02/95   claims 151-153 elected.
P007D 10/23/95   Amendment, adds claims 166-169; argues references
P007D 07/1/96   Amendment, adds claims 170-182, all directed to PLL on memory.
P007D 08/29/96   Notice of allowability (all claims)
P007D 02/7/97   Notice of abandonment
P007DC   11/15/96   Filed
P007DC   12/11/96   Preliminary Amendment
P007DC   481 claims 1-15
P008D 03/17/95   Amendment adds 151-158
P008D 11/21/95 Amendment adds 159-160
P008D 07/01/96   Amendment adds 161-166
P008D   Abandoned
P009D 06/04/93   Amendment adds 151-155
P009DC Amendment after final from prior application
P009DC   129 patent.
P010D 01/06/95   Amendment adds claims 151-168; prosecuted to final
P010DC   06/23/95   Preliminary Amendment 6/23/95. Claims 151-168 canceled.
Claims 169-185 added.
11/27/95 restriction into device ID, address range, access time claims.
P011D 03/05/92   Preliminary amendment adds Claims 151-156
P011D 575 patent claims 1-6
P012D 11/04/92   Amendment adds claims 151-163
P012D   703 issued claims 1-13
P013D   06/28/93   Preliminary amendment added claims 151-163.
P013D 02/02/95   -   Amendment adds new claims 164-169
P013D 385 claims 1-6
P014D 05/17/93   Preliminary amendment adds claims 151-164
P014D 01/11/95   Amendment 1/11/95 cancels claims 114-123, 155, 157-64, adds claim 165
(dep on 151); amends remaining claims.
P014D   717 claims 1-6
P043D 05/24/95   Preliminary amendment adds 151-165
P043D   Restriction
P043D   334 claims 1-5

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Arrived
December 29, 1997
Time period until: February 28, 1998

Filing Date: May, 28, 1997   Business Number: A 91/97-1.2
IPC: A61K   (indicate in all petitions)
To Immuno Aktiengesellschaft
In *CW* A-1221 Vienna
Attention: H. Pawloy; H. Sonn, A. Weinzinger, P. Pawloy *Vienna
Your Reference: 33052

Petition of July 17, 1997

1. PRELIMINARY NOTICE

On the basis of the results of the Preliminary Examination undertaken, in accordance with Section 99 of the Patent Law, you are invited--if the application is to be pursued further, to express your opinion in this regard, within two months after the delivery of the Preliminary Notice, and

o   Produce the uniformity of the application (Section 88, Patent Law)
x   Eliminate the deficiencies of the application
o   Appropriately carry out the corrections indicated in the enclosure -
o   Taking into consideration the criticism, present the following documents in duplicate:

    x   New patent claims
    o   A new description amendment/new description page(s)
    o   A new description introduction/adapted description
    o   A (new) cover sheet (Form PA 3 1)
    o   A (new) summary

o   Present the proper drawings
o   Thoroughly substantiate the invention characteristics with regard to the proven state of the art
o   Prove the priority right with regard to the literature that has appeared in the meantime (Section 95, Paragraph 3, Patent Law)

If the requests made are not complied with during this period or if an opinion or requests for an

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extension of the deadline are not submitted, then the application is considered to have been withdrawn. This legal conclusion is no longer in force, if within four months after the expiration of the deadline, the requests made are complied within or the opinion with regard to the Preliminary Notice is subsequently submitted and a fee is paid, in the amount of the application fee, to Postal Account No. 5,160,000 of the Patent Office. The request for an extension of the deadline is subject to a procedural fee in the amount of 170 S, which may not be paid in revenue stamps but rather must be paid to the Postal Account of the Patent Office. If a numbered paving-in slip of the Patent Office is used, payment of the aforementioned fees can be proved by submission of the order confirmation either in the original or in a copy; otherwise the original payment or remittance proof is to be presented.

The opinion and the enclosures are subject to stamp duty. The request for an extension of the deadline has to be stamped as a petition.

Attached are the following:

Description PAZ 25636 , Patent Claims PAZ

Description PAZ , Patent Claims PAZ

Sheet drawings PAZ ,

Summary PAZ , for use and resubmission

o Form PA 3 1, in duplicate   o   Form PA 3h   o   Attorney list

Result of the Preliminary Examination, on the reverse side of the page

Austrian Patent Office

Technical Department XVI

Vienna, December 12, 1997

Dipl. Ing. Wolf

917/97

Material prejudicial to novelty could not be determined. With regard to the known state of the art, US Patent No. 5,571,784 A (Behringwerke, 1996), dealt with in the description introduction, was taken into consideration.

Patent Claims 1-22, which is under consideration, should be written in the sense of Section 10, Paragraph 1, PGMMV.

Patent Claims 23 and 24 cannot be granted, since, on the one hand, they do not contain any detailed data on the field of application of the drug and thus do not correspond to the regulation of Section 91, Paragraph 1, Patent Law, and since on the other hand, in accordance with their vague content, they concern merely repetitions from the previous claims.

In the description, only published publications may be cited (see Description, page 8).

In this regard and with regard to other places in need of improvement in the application documents under consideration, reference is made also to the pencil remarks in the accompanying official copy.

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